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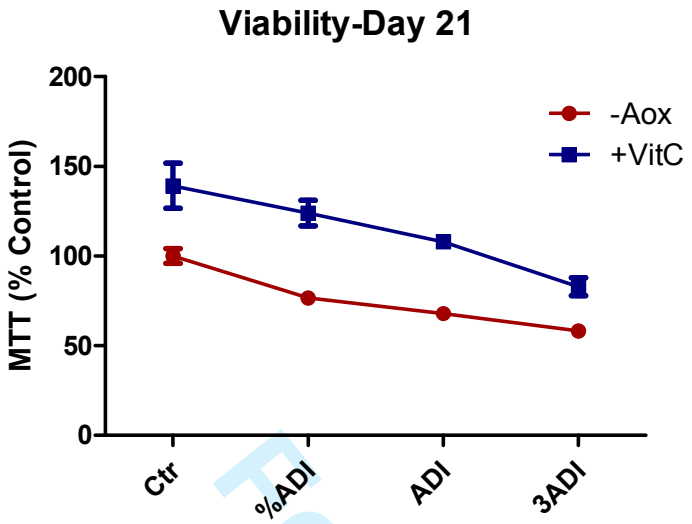

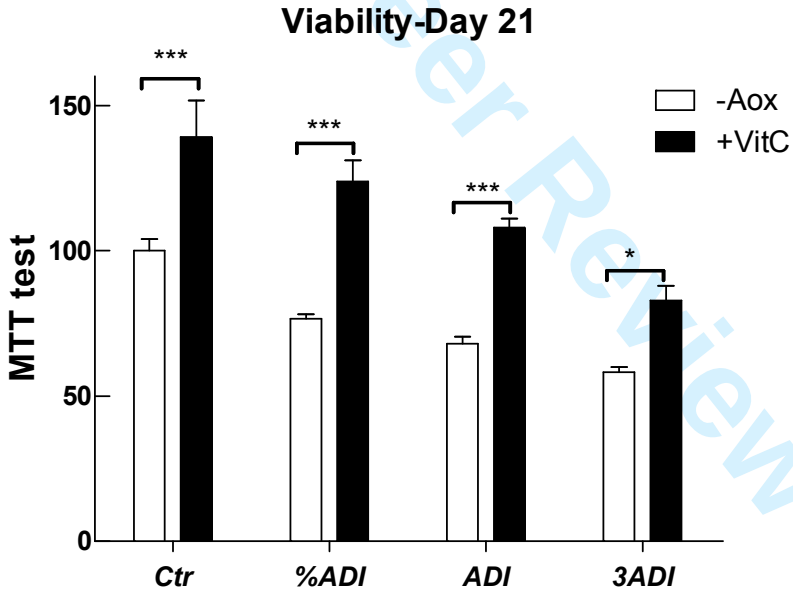

Low-dose pesticide mixture induces senescence in normal mesenchymal stem cells (MSC) and promotes tumorigenic phenotype in premalignant MSC.

Mazene Hochane[1,2], Valerie Trichet[3,2], Claire Pecqueur[1,2], Pierre Avril[3,2], Lisa Oliver[1,2,4], Jerome Denis[1,2], Régis Brion[3,2,4], Jérôme Amiaud[3,2], Alain Pineau[5,2], Philippe Naveilhan[6,2], Dominique Heymann[3,2,4], François M. Vallette*[1,2,7], Christophe Olivier*[1,2].

[1]Centre de Recherche en Cancérologie Nantes Angers UMR INSERM 892, CNRS 6299, Université de Nantes, Equipe Ligue 2010, 44007 Nantes, France.

[2]Université de Nantes, Faculté de Médecine, 44007 Nantes, France.

[3]INSERM, UMR 957, Equipe Ligue 2012. Pathophysiology of Bone Resorption Laboratory and Therapy of Primary Bone Tumours, Faculty of Medicine, University of Nantes.

[4]CHU de Nantes, 44093 Nantes, France.

[5]Pharmacology and Toxicology Department, University Hospital of Nantes, France.

[6]INSERM UMR 913, IMAD, France.

[7]LaBCT, Institut de Cancérologie de l'Ouest, 44805 St Herblain cedex, France.

Corresponding authors

Dr. Francois Vallette francois.vallette@univ-nantes.fr
Dr. Christophe Olivier Christophe.olivier@univ-nantes.fr

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AUTHOR CONTRIBUTION

CO and FMV developed the concepts and designed the experiments; MH performed in vitro experiments with the help of LO; CP and MH performed the SeaHorse experiments and analyses. VT, PA, JD, RB and JA performed in vivo experiments and analyses. DH and PN help to design in vivo experiments and to discuss results. MH, CO and FMV wrote the paper; all authors analysed results, wrote the methods section and edited the manuscript

ABSTRACT:

Humans are chronically exposed to multiple environmental pollutants such as pesticides with no significant evidence about the safety of such poly-exposures. We exposed mesenchymal stem cells (MSC) to very low doses of mixture of seven pesticides frequently detected in food samples for 21 days *in vitro*. We observed a permanent phenotype modification with a specific induction of an oxidative stress-related senescence. Pesticide mixture also induced a shift in MSC differentiation towards adipogenesis but did not initiate a tumorigenic transformation. In modified MSC in which a premalignant phenotype was induced, the exposure to pesticide mixture promoted tumorigenic phenotype both *in vitro* and *in vivo* after cell implantation, in all nude mice. Our results suggest that a common combination of pesticides can induce a premature ageing of adult MSC, and as such could accelerate age-related diseases. Exposure to pesticide mixture may also promote the tumorigenic transformation in a predisposed stromal environment.

INTRODUCTION

Pesticides play an important role in food supply protection and disease control, but there is a growing body of evidence that they are also harmful to human health under both occupational and non-occupational situations. Professional exposure to pesticides seems to be associated with higher incidence of cancers[1-3], endocrinal disturbances[4], and neurological degeneration[5] through direct contact with users or indirect transmissions to descendants[6, 7].Exposure to pesticides is a major public health issue because of their large amount used worldwide (estimated by millions of tons[8]). In the U.S, approximately 0.5 billion kilograms per year of pesticide active ingredients are used, and over different 17,000 pesticide products are being marketed[9]. Their intensive use in agriculture implies also dissemination to food and thereby to the general population. The pesticide quantities are limited to a theoretical safety threshold for humans, called the acceptable daily intake (ADI). However, even if the residual content of pesticides in food was lower or equal to their ADI values [10], The exposure of the population to these small quantities is constant and chronic and very few studies have evaluated its eventual risk. In addition, pesticides are generally applied in mixtures and thus several residues of molecules of different chemical families are present in food or water. In Europe, EFSA reports have shown that about 27% of fruits and vegetables were contaminated simultaneously with heterogeneous pesticides species [10, 11]. Thus, the risk of multiple exposures should be more accurately predicted by the evaluation, on a long-term basis, of mixtures of pesticides of different chemical families. However, there is no scientific evidence about the safety of the combination of such small residual quantities during a long-term exposure. Besides, this complex exposure might lead to insidious additive effects significantly different from those observed with a single type of pesticide [12].

We postulated that disturbances in cellular homeostasis may be induced by pesticides and would constitute their primary action and that these effects could be restricted to some organs/tissues. Hematopoietic tissue is a main site of exposure to pesticides. The chronic

exposure to high doses of mixtures of pesticides induced a direct hematotoxicity in the hematopoietic niche and led to hypoplastic marrow[13]. Mesenchymal stem cells (MSC) are important components of the hematopoietic niche and are necessary for niche homeostasis and their absence reduces hematopoietic stem cell development and repopulation [14, 15]. MSC are also found in other compartments such as adipose tissue, skin, dental pulp and in the circulation after bone marrow rupture [16]. They have the capacity to differentiate into many cell types including osteoblasts and adipocytes [17]which enlarges their functional contribution. Apart from their physiologic roles, MSC may promote tumour growth [18-20] or metastasis [21].

The aim of the study was to investigate the impact of long-term exposure of MSC to mixture of pesticides of different chemical families at low concentrations (nanomoles to a few hundred nanomoles range) extrapolated from the residual quantities found in the western European diet. For this purpose, we studied *in vitro*, the effect on MSC of a mixture of seven pesticides (Chlorpyrifos-Ethyl, Dimethoate, Diazinon, Iprodione, Imazalil, Maneb and Mancozeb) frequently detected in food samples[10].We found that this combination induces senescence *in vitro* and enhance tumorigenesis *in vivo*. Our results suggest that the combination of small amounts of pesticides could accelerate age-related diseases in healthy MSC and favour the onset of cancer in predisposed MSC.

MATERIAL AND METHODS

Materials

All pesticides (Chlorpyrifos Ethyl, Dimethoate, Diazinon, Iprodione, Imazalile, Maneb and Mancozeb) as well as the senescence cell histochemical staining kit (SA-beta-Galactosidase) were obtained from Sigma Aldrich (St. Louis, MO, USA). Foreskin-derived human fibroblasts were obtained from the Department of Pediatrics at “Hôpital Mère et Enfant de Nantes”. Human microvascular endothelial cells (HMVEC) were kindly provided by Dr

François Paris (INSERM-U892, Nantes, France). Mesenchymal stem cells (MSC) were obtained from human bone marrow aspirates which were kindly provided by Drs Philippe Rosset and Louis-Romée Le Nail from Tours University Hospital (France) during orthopedic surgical procedures. Oral consent was obtained from informed patients in accordance with French law (Art. L. 1245-2 of the French public health code, Law n° 2004-800 of 6 August 2004, Official Journal of 7 August 2004). At least five batches of MSC obtained from different young and healthy donors (aged of 23-35 years) were used. MSC were characterized as previously described [22-24]. Briefly, the surface markers including CD34, CD45, CD73, CD90, and CD105 and the differentiation capacity towards three lineages (osteoblast, adipocyte, chondroblast or myoblast) were assessed and confirmed as recommended[25].Cell media: Alpha-MEM, DMEM, RPMI-1640, and fetal calf serum (#10270) were fromThermoFisher Scientific (Villebon-sur-Yvette, France). Transformed mesenchymal stem cells were kindly provided by the laboratory of Dr Tim Fenton (UCL Cancer Institute, London, UK). MesenCult and the corresponding serum were from StemCell Technology (Grenoble, France). Medium and supplements for HMVEC (EBM-2) were from Lonza (Viviers, Belgium). The references of the antibodies used in this study are listed in Table S1.

Pesticides-mixture doses and preparation

The doses of pesticides used were extrapolated, for each pesticide, from three values. First, the high Nutritional Daily intake (hNDI), calculated for the French population of all-age, based on the method of the EFSA [11]. hNDI are the very low doses of pesticides used in our study (3 to 220 times less than the ADIs). The next doses are the international Acceptable Daily Intakes (ADI), specified by the WHO or the European Commission, representing the threshold of safety in humans for lifetime exposure. Last, a value of 3 times the ADI (3ADI) that we used as a positive control.

For the hNDI, the method in which EFSA determined chronic exposure is comparable to the calculation of the Theoretical Maximum daily Intake (TMDI) according to the following equation:

$$TMDI = \sum MRLi \times Fi$$

MRLi: Maximum residue level for food commodity I; Fi: Food consumption of food commodity i

In order to introduce a more realistic estimation of the exposure to a pesticide, the EFSA replaced the MRLi values by the relevant residue concentration obtained on analyzed samples. The calculated TMDI results were reported separately for each pesticide and for 27 different diets. The highest estimated exposure for each pesticide, expressed as percent of the ADI, is reported for each diet. In France, three groups of diets were represented: Infant, Toddler and general population according to the EFSA report 2010[10]. We calculated an average value (in % ADI) that we converted to mg/Kg.bw/day considering the ADI value for each pesticide. For the three values of hNDI, ADI and 3ADI, a further conversion has been made in order to extrapolate the aforementioned doses (estimated in mg/Kg body weight) to an in vitro model. In general, the amount of pesticide ingested daily is rapidly absorbed and rapidly eliminated (in whole or in part) by the body. We considered the case of a total absorption of this ingested amount and then its dilution in 5 liters of blood in a subject of 60 Kg, in order to obtain the blood concentration (mg/l) to which the various organs could be theoretically exposed. Finally, from the molar mass of each pesticide, we calculated a concentration in µmole/L.

Pesticides were dissolved in DMSO and mixtures were prepared at the three aforementioned doses. Whatever the dose applied, the maximal volumes of DMSO ± pesticides added to the media did not exceed 1/1000 (v/v) of the medium. Throughout the study, cells were treated with pesticides mixture for 21 days and media were changed every three days.

Cells Culture and treatment

Except for the experiments on MSC differentiation, MSC were cultured at 2000 cells/cm2 in alpha-MEM modified with ribonucleosides and deoxyribonucleosides and supplemented with fetal calf serum (FCS) (10%) and 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.2 ng/mL of FGF2 in an atmosphere of 5% CO2 and 95% humidity at 37°C. MSC cultures were used between passages 4 and 7. Modified MSC (we called Transformed MSC or tMSC) were cultured in complete MesenCult medium (StemCell technology). Fibroblasts were cultured in RPMI-1640 medium and supplemented with FCS (10 %) and 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (used between passage 4 and 50). Human microvascular endothelial cells (HMVEC) cultures were supplemented with standard commercial medium for endothelial cells (EBM-2) (Lonza). Pesticide mixture at doses of hNDI, ADI or 3ADI were dissolved in DMSO and added to the cell media, over the 21-day exposure. DMSO doses versus media did not exceed 1/1000 (v/v) for control and pesticides-treated cells. In some experiments, the pefithrin-α (10 µM) (Sigma) was used from day 7 until the end of the experiment. Media were replaced every 2-3 days.

Mitochondrial stress analysis

The mitochondrial stress and the glycolytic capacity of MSC were analyzed by the Seahorse XF24 Flux Analyzer (Seahorse Bioscience, Copenhagen, Denmark). Briefly, at day 7 and day 21 after exposure to the pesticide mixture, cells were trypsinized and reseeded at 2x104 cells /well and left overnight without the pesticide mixture to adhere before the analysis. Cells were equilibrated for 1 h at 37 °C in bicarbonate-free DMEM (Sigma) supplemented with 25 mM glucose, 1 mM pyruvate and 2 mM Gln, pH was adjusted to 7.3 with NaOH before the analysis. To determine mitochondrial parameters, Oxygen Consumption Rate was measured at baseline and after respective addition of oligomycin (0.75 µM), CCCP (1.5 µM), rotenone (1 µM) and antimycin A (1 µM). All measurements were done in five wells per condition.

Three to five independent experiments were conducted. Respiratory spare capacity was calculated as maximal OCR, after oligomycin and CCCP injections, minus basal OCR. Coupling efficiency corresponded to OCR inhibition by oligomycin. The basal glycolytic capacity was extrapolated from the extracellular acidification rates values (ECAR, ΔpH/min) of cell media before the addition of the aforementioned drugs.

In vitro MSC differentiation

MSC were seeded in 24-well plates at 3000 cell/cm2 and incubated with osteogenic differentiation medium (MSC Osteogenic bullet kit, Lonza) according to the description of the manufacturer, with or without pesticide mixture. Cells were also treated with pesticide mixture in their normal medium (non-differentiation condition) for 21 days. At the end of the experiment, mineralization was detected by Alizarin Red staining (Sigma) and quantified after the solubilization of the dye as previously described[23]. For further analysis of the expression of runt-related transcription factor 2 (RUNX2) and alkaline phosphatase (ALP) transcripts, total RNA was extracted using NucleoSpin RNA II (Machery-Nagel, Düren, Germany) at days 7,14 and 21. Reverse transcription (RT) was performed using 0.2 µg of total RNA and ThermoScript RT (Invitrogen Life Technologies). Then 20 ng of cDNA were amplified using the IQ SYBR Green Supermix (Bio-Rad) with primers. Gene names and primer sequences are for RUNX2: forward primer GTGCCTAGGCGCATTTCA and reverse primer GCTCTTCTTACTGAGAGTGGAAGG and ALP forward primer AACACCACCCAGGGGAAC and reverse primer GTAGCTGTACTCATCTTCATAGGC and RLP19 forward primer GCTCTTTCCTTTCGCTGCT and reverse primer CATTGGTCTCATTGGGGTCT and GAPDH forward primer TGGGTGTGAACCATGAGAAGTATG and reverse primer GGTGCAGGAGGCATTGCT.

Quantitative analysis was performed with the iCycler iQ Real-time PCR Detection System (Bio-Rad). Relative fold change of gene expression was calculated following the delta delta

Ct method[26]. The reference genes RLP19 and GAPDH were used for normalization. For the adipogenic differentiation of MSC, cells were seeded in 24-well plates and on 4 chambers glass slides (Labtek, Dominique Dutscher, Brumath, France) at 2x104 cells/cm2 and incubated with MSC Adipogenic Bullet kit (Lonza) for 21 days. The same scheme of pesticides exposure was pursued as for osteogenic differentiation. Adipocytes were further stained with Oil Red O staining (Sigma), and counterstained with hematoxylin. For 24-well plates, images were acquired by Arrayscan VTI HCS reader (ThermoFisher Scientific) and for the slides we used the whole-slide scanner NanoZoomer 2.0-HT (Hamamatsu).

In vivo MSC injection

Animal handling and surgery were conducted in accordance with the European Community Guidelines (2010/63/EU) for the care and use of laboratory animals. An animal experimentation protocol was prepared, submitted and approved by the regional committee on animal ethics named CEEA.2013.4, with project authorization number 2013.4. Nude female mice of 4 weeks old were obtained from Centre d'Elevage Janvier (Le Genest-Saint-Isle, France). They were kept in a pathogen-free barrier facility. They had access to food and water ad libitum. The cell implantation was done as previously[22]. tMSC-4hits and tMSC-1hit were treated with DMSO or with pesticides-mixture at the dose of ADI for 21 days. The day of the implantation, cells were trypsinized, filtered to dissociate cell clumps and counted. Four million cells were then implanted in the paratibial zone. Animals were injected bilaterally with either pesticides-treated cells (tMSC-4hits in the right leg and tMSC 1hit in the left one) or DMSO-treated tMSC. Mice were followed up until tumor development and were euthanized when tumor volume reached 500 to 1500 mm3. Tumor volume was calculated according to the following formula: $(l^2xL)/2$ where l and L are the smallest and largest diameters respectively.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed when appropriate using one-way or two-way ANOVA followed by Tukey or Dunnett's multiple comparison tests. For in vivo comparison "Gehan-Breslow-Wilcoxon Test" was used. Statistics were run with GraphPad Prism®. Differences with $P<0.05$ were considered statistically significant.

Miscellaneous

For Cell count and viability, SA-Beta-galactosidase activity, Measurement of reactive oxygen species (ROS), Immunoblot, immunofluorescence and caspase activity, Mito Tracker staining, Anchorage-independent cell growth, Histology and flow cytometry analysis, see supplemental data; supplemental methods.

RESULTS:

Specific induction of senescence in MSC by an environmental combination of pesticides.

In order to mimic the heterogenic human exposure, 7 chemicals of four structural and mechanistically distinct categories were elected (3 organophosphorus compounds, 2 Dithiocarbamates, 1 Dicarboximide and 1 imidazole).We used three different mixtures of the seven pesticides at doses calculated as detailed in "Pesticides-mixture doses and preparation": the lowest doses (hNDI) are extrapolated from the published residual quantities found on foodstuff [10](Fig.S1 and Table.S2). The effect of the three doses of pesticide mixture was tested after 21-day exposure on various types of human adult cells such as fibroblasts, human lung microvascular endothelial cells (HMVEC) and mesenchymal stem cells (MSC). As shown in Fig.1A and B, no effect on cell proliferation and/or death was observed under all conditions for fibroblasts and endothelial cells. In contrast, a 3-week exposure to pesticide mixture of MSC decreased their proliferation as a function of dose, as shown by BrdU assay (Fig.1C) and cell count (Fig S2A) without affecting their viability, evaluated by PI staining (Fig.1C) and MTT assay (Fig.S2A). Of note, individual exposure of MSC to each pesticide did not significantly alter cell proliferation with doses of hNDI and ADI **(**Fig.S3**).** These

results support our contention of a theoretical additive response of our pesticide combination that is not the mere sum of individual effects and shows specific cellular response. MSC cell death did not seem to be affected by the treatment at any combination concentration and no apoptosis was observed at day 21 as no caspase-3 activity was detected (Fig.S4A). The expression of some proteins of the BCL2-family was deregulated (i.e. BAX and MCL-1 expressions were decreased and BCL_{XL} was increased) which suggested an increased resistance to apoptosis (Fig.1D and Fig.S2B). However, since the resistance to cell death in undifferentiated MSC is usually high[27], the consequence of the treatments on the threshold to apoptosis might not be efficient. Autophagy and senescence could protect the cell from the toxicity of external stress such as pesticides [28, 29]. Basal autophagy is already important in MSC [29], and no increase of the cleaved forms of LC3 and degradation of p62 independently of the administered doses was observed (Fig.1E and Fig.S4C). Next, we determined if the reduced cell number was due to senescence. An increase of senescent cells was established in MSC in a dose-dependent manner (Fig.1F). MSC senescence was important even at very low dose of pesticide mixture and ranged from 30% with hNDI to about 50% with 3ADI. Of note, this effect was specific to MSC at these doses as we did not observe any induction of senescence in fibroblasts or in endothelial cells after 3-week exposure to the pesticide mixture (Fig.1G and H and Fig.S5, upper panel).

Pesticide mixture does not induce a replicative senescence in MSC

Cellular senescence may be replicative (telomere-dependant) or stress related. To further study the pesticide-induced senescence, we used, in addition to naïve MSC, the modified human adult MSC (tMSC) with genetic alterations described by Funes *et al.*,[30] (Fig.S6). In naïve MSC, the pesticides induced an upregulation of $p21^{wafl}$ but not in p53 expression at day 21 or earlier (Fig.2A and Fig S4D), while $p16^{INK4a}$ was not detected by Western blot in these cells under our conditions (Fig.S4B). In addition, the inhibition of the transcriptional activity

of p53 by pifithrin-α (10 μM from day 7) (Fig. S5, lower panel) or its invalidation in tMSC-2hits (Fig.2B) completely abrogated the pesticides-induced senescence. In tMSC-1hit, the ectopic expression of hTERT extends MSC lifespan in culture as previously described [30]. In these cells, pesticides were able to induce dose-dependent senescence (from 15 % with hNDI up to 40% with 3ADI) although with less efficiency than that observed in naïve MSC. Of note, $p21^{wafl}$ was upregulated in these cells (Fig.2C). Altogether, this suggests that pesticide-induced senescence in MSC was p53 but not telomere dependent.

Pesticide mixture induces a stress-related senescence in MSC.

Oxidative stress is one of the classical initiators of senescence[31]. Pesticides can also induce an oxidative stress in many cell types [32-35]. To analyse the effect of the pesticide combination, the generation of reactive oxygen species (ROS) was analyzed at day 7, 14 (Fig. S7A) and day 21 (Fig.3A). Our combination was able to generate a dose-dependent increase of continuous ROS production in MSC even with the lowest concentrations used, persistent until day 21(Fig.3A). ROS production in fibroblasts and endothelial cells was moderately increased only at highest concentrations of pesticides in endothelial cells (3ADI, Fig.3A) while no increase was detected in fibroblasts at day 21(Fig.3A).Of note, the basal levels of ROS were nonetheless superior in endothelial cells and fibroblast compared to MSC. Since the induction of ROS by our pesticide combination appeared to be specific to MSC, we investigated further its mechanisms. The expression of various antioxidant enzymes was evaluated by immunoblots in MSC and fibroblasts (Fig.3B) after 21-day exposure. In MSC, hNDI dose induced an upregulation of the mitochondrial enzymes superoxide dismutase SOD2 and glutathione peroxidase GPX1, whereas higher doses (ADI and 3ADI) led to a more potent response with an additional increase in both cytosolic proteins GPX2 and catalase expression. These effects were not related to a deterioration of the mitochondrial structure as shown by the staining of functional mitochondria and the steady levels of citrate-synthase, a

marker of mitochondrial biogenesis (Fig.S7B). In fibroblasts, the enzymes were constitutively expressed in control cells and no significant changes in the expression of SOD1/2, GPX1/2 or catalase were seen (Fig. 3B). This antioxidant profile was consistent with the ROS production profile. Moreover, vitamin C reduced ROS production in MSC treated or not with the pesticide mixture at day-4 (80%) without affecting their viability (Fig.S7C).Treatment with vitamin C, starting on day 7 during the 3-week exposure to pesticide mixture reversed the pesticides-induced senescence in MSC and attenuated the expression of $p21^{waf1}$ and p53 (Fig. 3C) without affecting cell viability (Fig.S7C). Altogether, these results indicate that our pesticide mixture induced specifically a stress-related senescence in MSC that can be overcome by vitamin C.

Exposure to Pesticides leads to early and late respiratory changes in MSC

It has been reported that exposure to some pesticides, especially organophosphates, disturbed the cell respiratory system[36]. Moreover, oxidative stress[37] as well as induction of senescence[38] may alter the cellular metabolic capacities. So,we investigated the impact of our pesticide mixture on the mitochondrial respiratory profile of MSC and their basal glycolysis early on day 7 and on day 21. While a global reduction in both mitochondrial capacity and glycolysis was observed at day 7 (Fig.S8), the global metabolic profile was completely different on day 21. Basal mitochondrial respiration was restored at doses of hNDI and ADI and even increased in 3ADI-treated cells. A similar effect was observed with OXPHOS-coupled ATP production (Fig.4A). The expression of ATP synthase was also increased (Fig. 4B). However, pesticides-treated MSC showed an important use of their spare capacity, which is the extra capacity available in cells to produce energy in response to increased stress (spare capacities were 38.10±5.6 in Control MSC versus 15.69±4.05, 19.93±5.14, 10.77±2.78 with hNDI, ADI and 3ADI respectively, $p<0.05$). Proton leak was unchanged. The basal glycolysis in MSC, a main energy source for MSC *in vivo*[39] was not

significantly different from control cells (Fig.4C). Altogether, the late respiratory features show an adaptation of MSC, despite the continuous presence of pesticides at this dose range, leading to an elevation of OXPHOS at 3ADI. Moreover, mitochondria functioned at full capability to ensure sufficient metabolic resources losing their spare capacity, which is necessary to face environmental stress and increased energetic demands.

Pesticides alters the differentiation in MSC

One important specificity of MSC is their capacity to differentiate into other cell types like osteocytes and adipocytes. However, little is known about the effect of pesticides, either in mixtures or alone, on the differentiation of MSC. A 21-day treatment with pesticide mixture did not alter the MSC stemness as they continued to express OCT-4 and SOX-2 (Fig S9A) with an increase in SOX-2 expression. We induced osteogenic or adipogenic differentiation in MSC in the presence of pesticides during 21 days. MSC showed lower tendency to osteogenic differentiation at low doses (hNDI and ADI), and this was significant with 3ADI as seen with Red-Alizarin staining (Fig.S9B). This was accompanied by a decreased expression of *RUNX2* transcript, a major transcription factor associated with the induction and commitment to osteogenesis, on day 7. In contrast, the expression of *ALP* transcripts was not significantly changed (Fig.S9C). On the other hand, the adipogenic differentiation was assessed and showed a significant dose-dependent increase in presence of pesticide mixture even with the lowest concentrations (Fig.5).

Pesticide mixture promotes tumorigenic phenotype in modified MSC

Pesticide exposure was associated with high risk of cancers [3, 40, 41], in addition, cellular senescence may constitute a permissive environment to cancer development[42]. To examine the capacity of the low-dose pesticide mixture to induce malignant transformation in MSC, we tested the anchorage-independent growth of MSC treated with the pesticide mixture for 21 days. Naïve MSC were unable to form colonies in soft agar whether they were pre-treated or

not with pesticide mixture (Fig.S10A and table.S3) which indicates that pesticide mixture alone could not initiate tumorigenesis in normal MSC. We looked further to see if the pesticide mixture could promote a tumoral phenotype in modified tMSC. For this purpose, tMSC which were transduced by 1 to 4 oncogenic hits were used. A 21-day pre-treatment with pesticide mixture enhanced the colony formation after 28 days in the soft agar assay (Fig.S10A and table.S3). However, this effect was not dose dependent but was already important at the very low dose of pesticides (hNDI).

In order to evaluate this effect *in vivo*, we treated both tMSC-1hit and tMSC-4hits either with DMSO or with our pesticide mixture at ADI dose. The cells were separately implanted bilaterally in *nude* mice paratibial zones. The first group was implanted with cells treated with DMSO (DMSO-group) and the second group with pesticide mixture-treated cells (ADI-group). Strikingly, only 3 of 9 mice showed tumour growth in DMSO-group all over the experiment while all the mice in ADI-group developed tumours ($p = 0.011$). In both cases, tumours were only observed with tMSC-4hits (Fig.6A and 6B), which corroborates the observation on tMSC clonogenicity *in vitro*. Furthermore, tumorigenesis showed accelerated kinetics of apparition in ADI-group compared to DMSO-group with onset after 160.14 ± 31.62 days versus 204 ± 22.52 days respectively (Fig.6C). Histological analysis of tumours obtained from the ADI-group, showed undifferentiated connective tissues with fibroblastic and mixoid zones (HE staining, Fig.6D). Within these pleomorphic sarcomas induced by ADI-treated MSC-4hits, specific staining against human Ki-67 revealed a high proportion (>13 % ± 6) of proliferating human cells (Fig.6D). *Ex vivo* culture of tumour cells were prepared and analyzed by flow cytometry for cluster differentiation markers (CD) that are characteristic of MSC. Cells derived from the tumours of ADI-group showed a CD expression pattern similar to MSC (negative for CD34 and CD45 and positive for CD44, CD73, CD90, CD105), the same pattern found in tMSC-4hits before any treatment (Fig.6E, Fig.S10C).

Altogether, this indicates that the 21-day exposure to pesticide mixture was sufficient to induce long-lasting phenotypic changes in tMSC-4hits exacerbating and accelerating their tumorigenic transformation.

DISCUSSION

The population is exposed in a chronic manner to multiple residues of pesticides belonging to diverse chemical families by, among others, the nutritional contamination. The EFSA report has shown that about 27% fruits, vegetables and commodities in Europe were contaminated with heterogeneous residues of pesticides simultaneously[10, 11]. The seven pesticides we chose (depicted in table S2) were among those frequently used and detected on food and commodities, according to the EFSA's report of 2010 and still feature in the recent report of 2013 (See supplemental Fig.S1). In addition, they may be simultaneously present on different fruits and vegetables (like apple, tomato, cabbage, lettuce and pepper) and some of them like mancozeb, diazinon, Chlorpyrifos and the dithiocarbamates were associated with hematologic disturbances in humans[43, 44] and in animal studies.[13, 45, 46] We used, for our *in vitro* model, an experimental estimation of the nutrition-conveyed concentrations of pesticides to which the population may be exposed considering the average of the highest calculated exposures of all-age French population according to the EFSA's method. The final *in vitro* concentrations correspond to those obtained through one-compartment pharmacokinetic model for hNDI, ADI and 3ADI doses. The selected hNDIs, with doses 3.15 to 220 times inferior than their chronic risk thresholds, i.e. ADIs, only correspond to the maximal risk related to food ingestion, while the major population is exposed to divers other sources (through household use, environmental contamination, drinking water…etc). In addition, active principles were used in this study while pesticides are usually combined with adjuvants that may increase their toxic effects[47].

One of our major observations is a dose-dependent induction of senescence in MSC after 21-day exposure to pesticide mixture at notional doses. This effect seemed selective to MSC but not to other cells such as endothelial cells and fibroblasts. On one hand, induction of senescence was important in MSC even with the lowest doses (about 30% cells) and was consistent with the dose-dependent decrease in number of proliferating cells at day 21, taking into consideration that the pesticide mixture did not alter the viability or induce death in MSC. On the other hand, pesticides were able to induce senescence and $p21^{WAF1}$upregulation in transformed MSC in which the human telomerase (hTERT) was constitutively expressed, but the percentages of senescent tMSC were less than those in naïve MSC. Although this may be due to the transduction of these cells, it may also suggest that only few MSC may have undergone a telomere-dependent aging. Altogether, our results indicate that pesticides-induced senescence was p53-dependent and mainly stress-related.

Pesticides are known to cause oxidative stress in different cell types[34] and this may be a possible starting point for senescence[31].The mixture led to a continuous dose-dependent ROS production in MSC whereas fibroblasts and endothelial cells appeared less sensitive. Interestingly, pesticides-treatment showed a rather dose-dependent activation of antioxidant enzymes; while hNDI upregulated mitochondrial SOD2 and GPX1, ADI and 3ADI doses were more aggressive and induced cytosolic enzymes too (GPX2, SOD1 and catalase). Nonetheless, it is important to note that only a high concentration of vitamin C (200 µM) was sufficient to reverse pesticide mixture-induced oxidative stress in MSC, while the plasma concentrations of vitamin C in the general population seem to vary, in function of age and health, between 31 and 44µM, with a recommended optimum level of about 50µM[48], suggesting that physiologic concentrations may not be sufficient to counteract the pesticides-induced senescence generated in MSC.

Pesticides exposure has been reported to induce metabolic alterations [36, 49]. After 21-day exposure to pesticide mixture, MSC adapted to the prolonged aggression as they restored basal glycolysis and showed even more important OCR and mitochondrial ATP production with an upregulation of ATP synthase. However, the decrease in mitochondrial spare capacity persisted. As this capacity can be used by the cell in order to face an environmental stress or increased energetic demands[50], the persistent reduction in MSC's spare capacity, with continuous exposure to pesticide mixture, suggests that MSC may become more vulnerable if further aggressions occur.

Exposure of MSC during 21 days to pesticide mixture did not seem to alter their stemness as MSC continued to express both OCT4 and SOX2 with even higher levels of SOX2 as seen by immunoblot analysis. Neither the impact of pesticide mixture on SOX2 expression nor a potential link between stemness markers and senescence in MSC are clear. However, in neural stem cells, SOX2 overexpression induced senescence [51].

On the other side, when MSC were committed to differentiation media, their differentiation potential of MSC into adipogenic or osteogenic lineage was disturbed by pesticide mixture. In fact, MSC showed lower tendency to differentiate into osteocytes while their adipogenic potential was raised. It is noteworthy that during aging, bone marrow is replaced by adipose tissue and shows progressive bone fragility [52]. Adipose replacement also plays an important role in haematopoietic alteration during aging [53].

Many epidemiologic reports associate pesticide exposure and cancers [3, 40, 41]. Exposure to our pesticide mixture was unable to initiate tumorigenesis in normal MSC *in vitro*. However, in modified tMSC developed by Funes JM *et al*[30], in which a stepwise oncogenic transformation was induced, the exposure to pesticide mixture increased the colony formation in tMSC-3hits and tMSC-4hits but not in tMSC-1hit expressing human telomerase (hTERT) only, as shown by soft agar assay. *In vivo* implantation in paratibial zone in nude mice

showed that 21-day pre-treatment with pesticide mixture at ADI dose shifted tMSC-4hits into more tumorigenic phenotype as tumours appeared in all mice implanted with ADI-treated tMSC-4hits compared to 3/9 mice injected with DMSO-treated tMSC-4hits. The onset of tumour growth was also earlier in ADI-group (169 days median) compared to DMSO-group (217 days median). It is noteworthy that in the study of Funes JM *et al*, tMSC-4hits did not show tumour growth when injected subcutaneously in nude mice after 6-month follow-up and a fifth hit was necessary to achieve their complete oncogenic transformation[30]. The tumour growth in DMSO-group found in our study may be due to a different site of implantation. It is important to point out that tMSC-1hit did not develop any tumours. The last observation is in line with the *in vitro* colony formation.

Human MSC-4hits pre-treated with pesticide mixture induced undifferentiated high-grade pleomorphic sarcomas, similarly to tumours induced in mouse following *p53* and *RB* deficiency[54]. Ex vivo analysis of tumour cells showed that tMSC-4hits treated or not with pesticide mixture remained undifferentiated and similar to parental cells (supplementary Figure S10B). MSC have been proposed as the cell of origin of several human soft tissue sarcomas including leiomyosarcoma and osteosarcoma but this was mostly proved in animal studies based on mouse MSC derived from p53 and/or RB deficient models[55, 56]. Our data using human premalignant MSC indicate that pesticide-stress may enhance tumorigenesis in already transformed cells by inducing additional step towards a complete malignancy.

In conclusion, we show that a mixture of pesticides that can be ingested on a daily basis may constitute an additional environmental factor favouring aging and aging-associated pathogenesis in normal adult stem cells and promote tumorigenesis in a predisposed stromal environment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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LEGENDS TO FIGURES

Fig.1, Effect of pesticide mixture on fibroblasts, endothelial cells and MSC. (**A**) Endothelial cells were treated or not with pesticide mixture for 21 days and cell growth was analyzed by MTT at day 21 (n=3). (**B**) Fibroblasts were exposed to pesticide mixture for 21 days and assayed as endothelial cells (n=3). (**C**) MSC proliferation in the absence or in the presence of pesticide mixture for 21 days. MSC were seeded at 2000 cells/cm^2 and treated with DMSO 1/1000 v/v (Ctr) or pesticides mixture for 21 days. Cell viability was analyzed by Propidium iodide (PI) staining ,and proliferation by BrdU incorporation the end of the experiment, n=3, * p< 0.05, **p<0.01 *vs* Ctr. (**D**) The expression of pro and anti-apoptotic proteins in MSC was analyzed by immunoblot after 21-day exposure to pesticide mixture (n=3). (**E**) LC3ß cleavage and p62/SQSTM1 degradation were detected by immunoblot analysis at day 21 after pesticide mixture treatment (n=3). (**F**) β-gal staining of MSC after 21-day exposure to pesticide mixture. The percentages of senescent cells were then determined. (n=3, p<0.01 *vs*Ctr). (**G**) Endothelial cells and (**H**) fibroblasts were treated with pesticide mixture for 21 days then β-gal activity was determined (n=3) by β-gal staining (n=3).

Fig.2 Pesticide mixtures induce p53-dependant but not telomere-dependant senescence in MSC. (**A**) Immunoblotting and semi-quantification of p21^{waf1} and p53 in MSC at day 21 after exposure to pesticide mixture (n=3). (**B**) β-gal staining and quantification in transformed tMSC-2hits (hTERT$^+$/p53$^-$) 21 days after pesticide exposure (n=3). (**C**) β-gal staining in tMSC-1hit (hTERT$^+$) in presence of pesticides at day 21. The percents of senescent cells in tMSC hTERT$^+$ cultures were assessed for each condition and the immunoblot of the expression of p21^{WAF1} at day 21 was analyzed (n=3, * p<0.05, ** p<0.01).

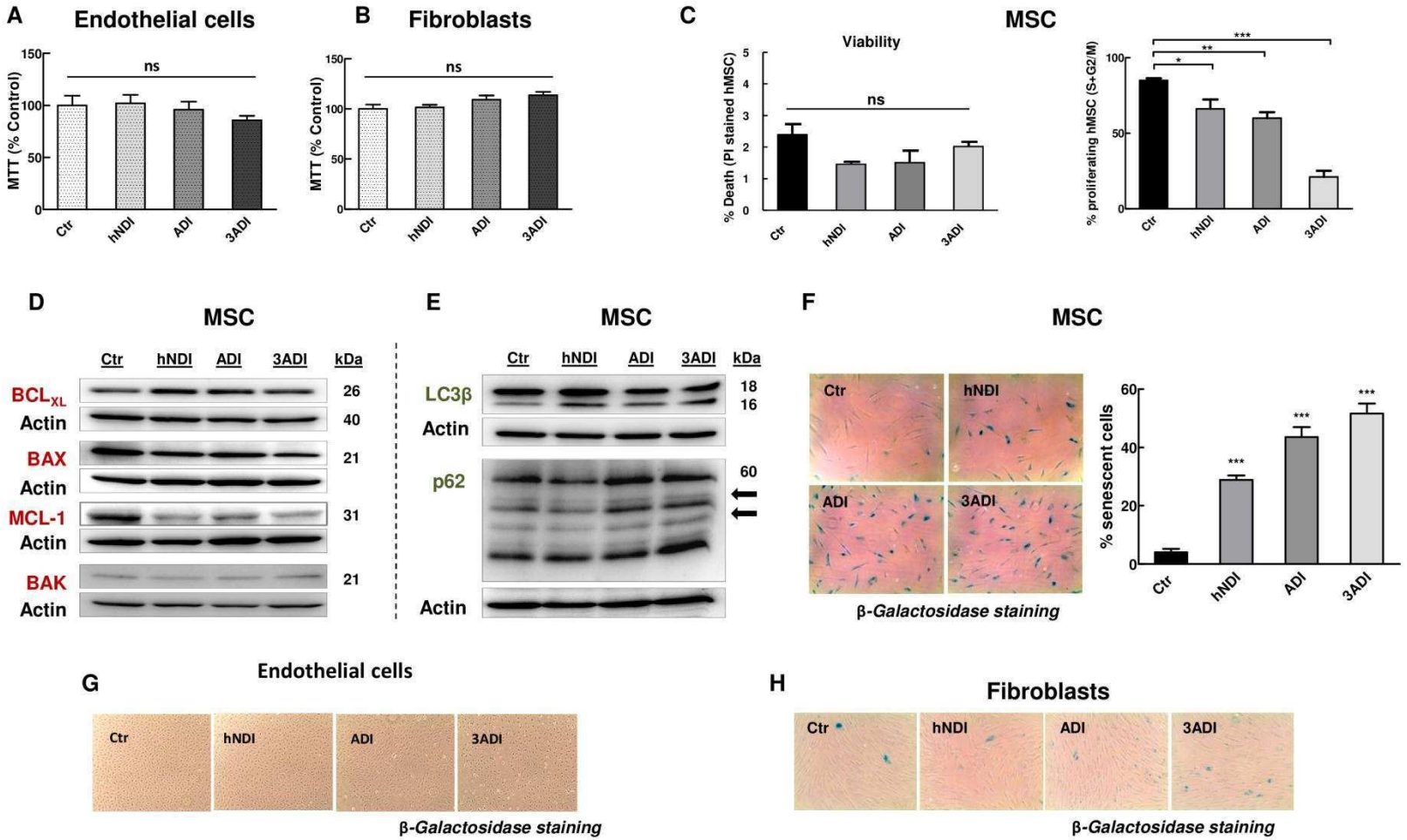
Fig.3 Pesticide mixture enhances ROS production specifically in MSC and oxidative stress reversion abrogates pesticide-induced senescence in MSC. (**A**) ROS production was analyzed in MSC, fibroblasts and endothelial cells using CMH_2-DCFDA at day 21 (n= 3,

*p<0.05, **p<0.01). (**B**) Expression of mitochondrial (SOD2 and GPX1) and cytosolic (SOD1, GPX2 and catalase) after exposure to pesticide mixture for 21 days by immunoblot in MSC (left panel) and fibroblasts (right panel) (n=3). (**C**) β-gal staining and quantification of MSC after 21-day exposure to pesticide mixture in the presence of vitamin C (200 μM) from day 7 (n=3) (upper images). ROS activity in MSC in presence of vitamin C at day 21 (***p<0.01 versus vitamin C treatment, $^{\#}$ p<0.05 Ctr versus pesticide-treatment, n=3), expression of $p21^{WAF1}$ and p53 under the same conditions (n=3).

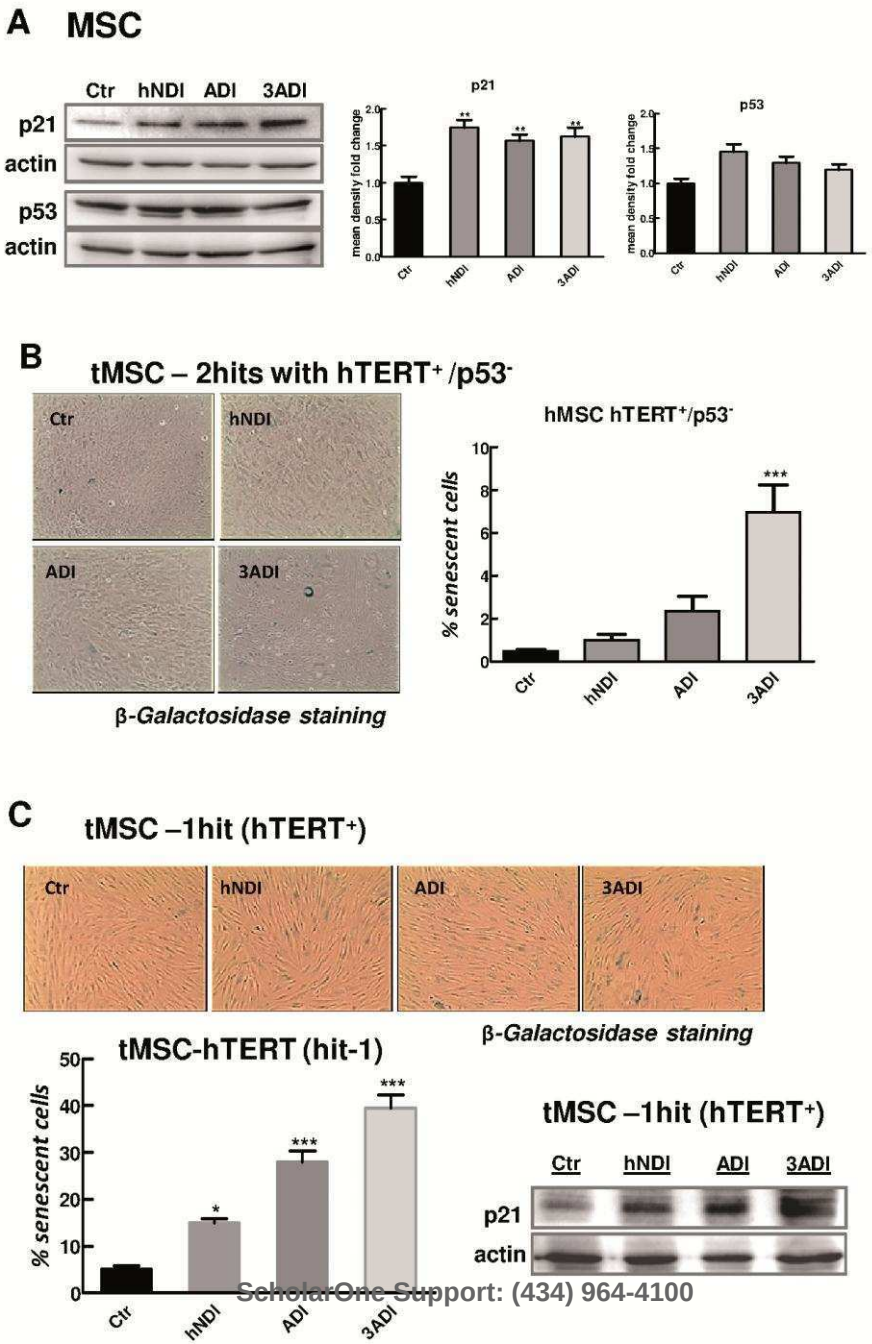
Fig.4 Pesticide mixture causes respiratory changes in MSC. (**A**) Mitochondrial respiration was assessed using the Seahorse XF24 Flux Analyzer. After 21 days of exposure to pesticide mixture, cells ($2x10^4$) were seeded in 24-well XF plate and the oxygen consumption rate (OCR) was measured before drug addition (basal respiration), mitochondrial ATP production was estimated after oligomycin treatment, proton leak was the result of the excess OCR between basal OCR and values after oligomycin addition. The % spare respiratory capacity was calculated after CCCP treatment. (**B**) The expression of ATP synthase (subunit-α) was analyzed by immunoblot at day 21. (**C**) Basal glycolysis was estimated from the ExtraCellular Acidification Rate (ECAR) before addition of drugs. (n=3, *p<0.05, **p<0.01).

Fig.5 Pesticide mixture promotes adipogenic transformation in MSC. After 1-week treatment with pesticide mixture, adipogenesis was induced or not in MSC in presence of pesticides. Fourteen days later, the cultures were stained with Oil-Red and counterstained with hematoxylin. The photos are representative pictographs of the different cultures (bar of 200 μm). The percent of adipocytes was calculated either manually or after scanning with HCS Studio software. Results are presented as % adipocytes. (n=3, *p<0.05, ***p<0.001)

Fig.6 Pesticide mixture promotes the tumorigenic phenotype of tMSC*in vitro* and *in vivo*. (**A**)Illustration of tumour growth in nude mice after paratibial implantation of tMSC-1hit or tMSC-4hits pre-treated for 21 days with DMSO (DMSO-group) or with pesticide mixture at ADI dose (ADI-group). (**B**) Tumour incidence in both groups (3 mice of 9 in DMSO-group and 8 mice of 8 in ADI-group, **p=0.011). (**C**) Kinetics of tumour apparition and growth in DMSO-group and ADI-group indicating the onset and the tumour growth in each mouse, mice were included when tumour volume attained 100 mm^3. (**D**) Histological analyses of tumours obtained from ADI-group showing soft tissue sarcoma (HE staining) and proliferating human cells specifically detected with anti-human Ki-67 (100µm). (**E**) Flow cytometry analysis of e*x vivo* cultures of tumour cells from both DMSO- and ADI-groups showing positive staining to MSC stemness markers.



Hochane et al. Fig.1



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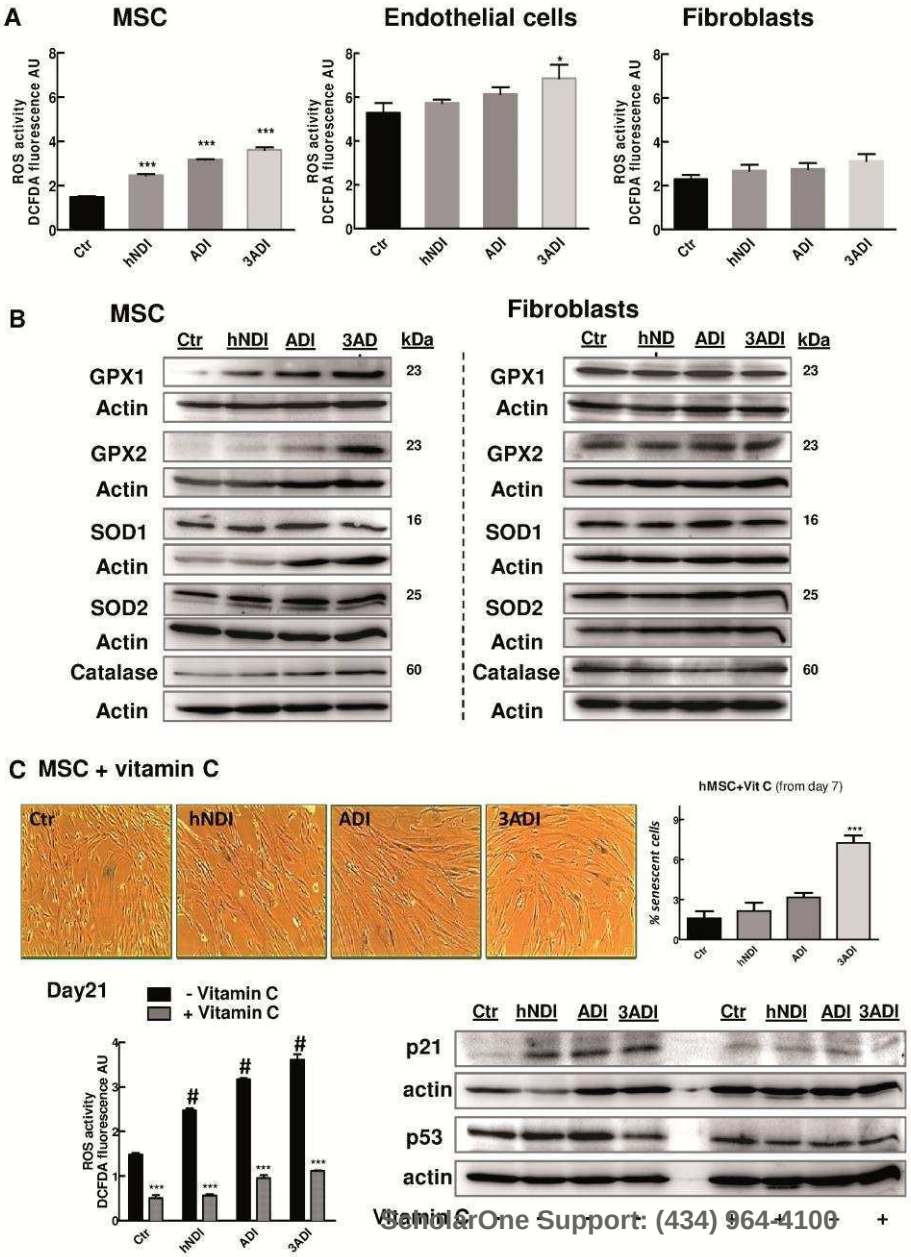

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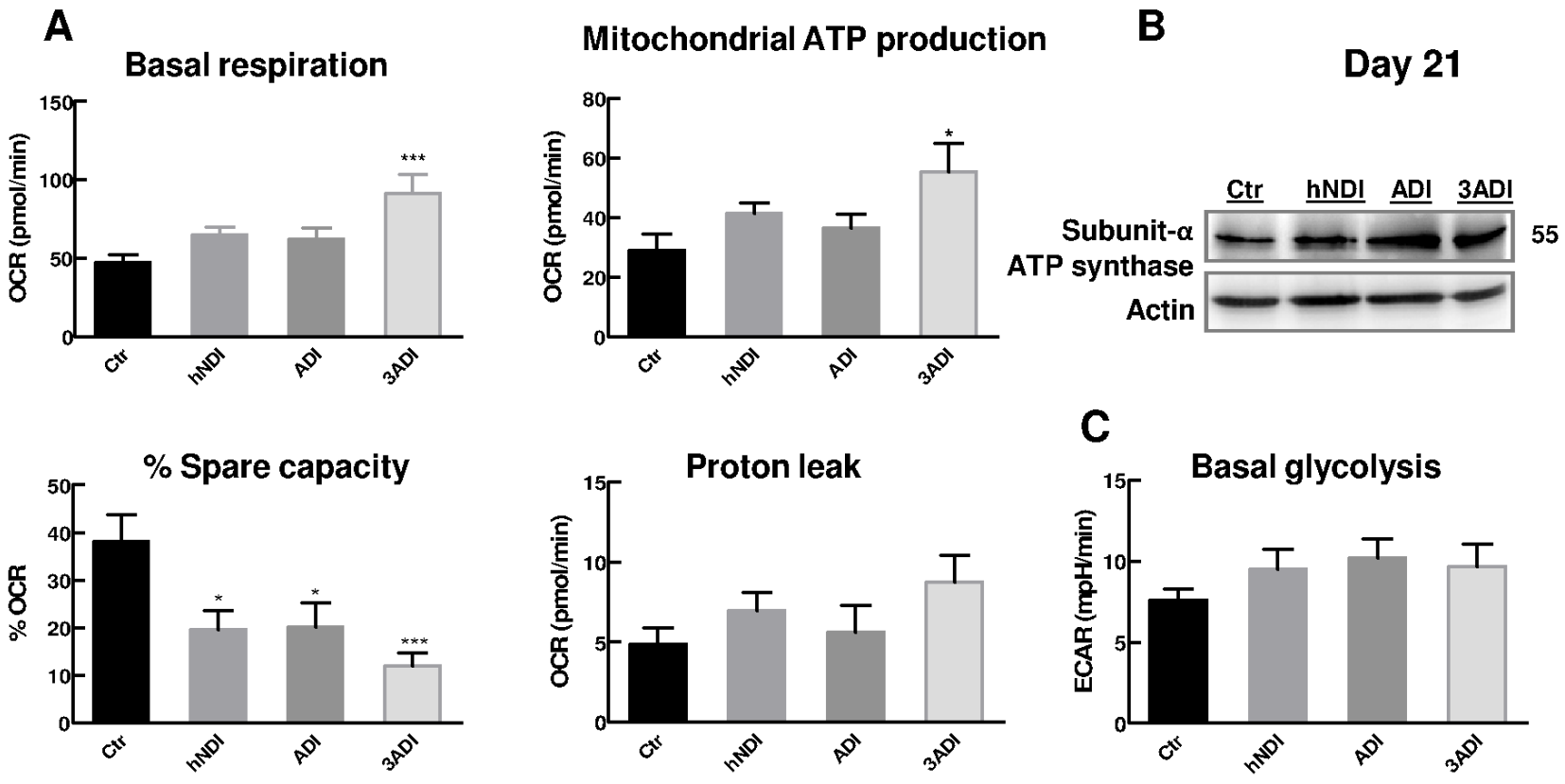

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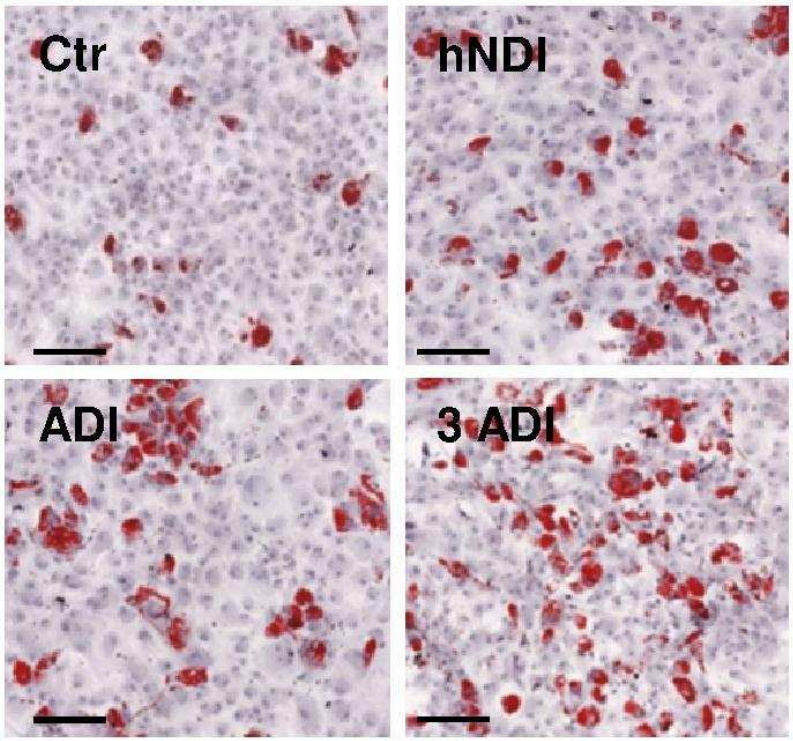
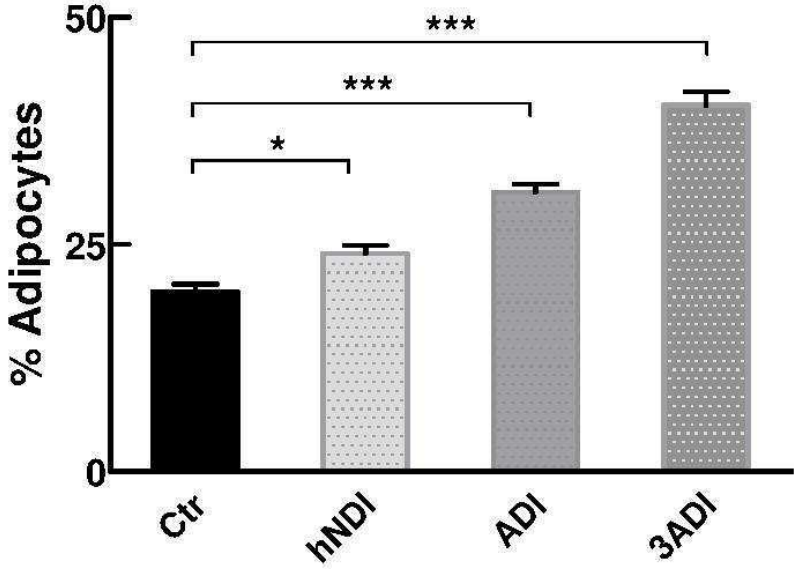
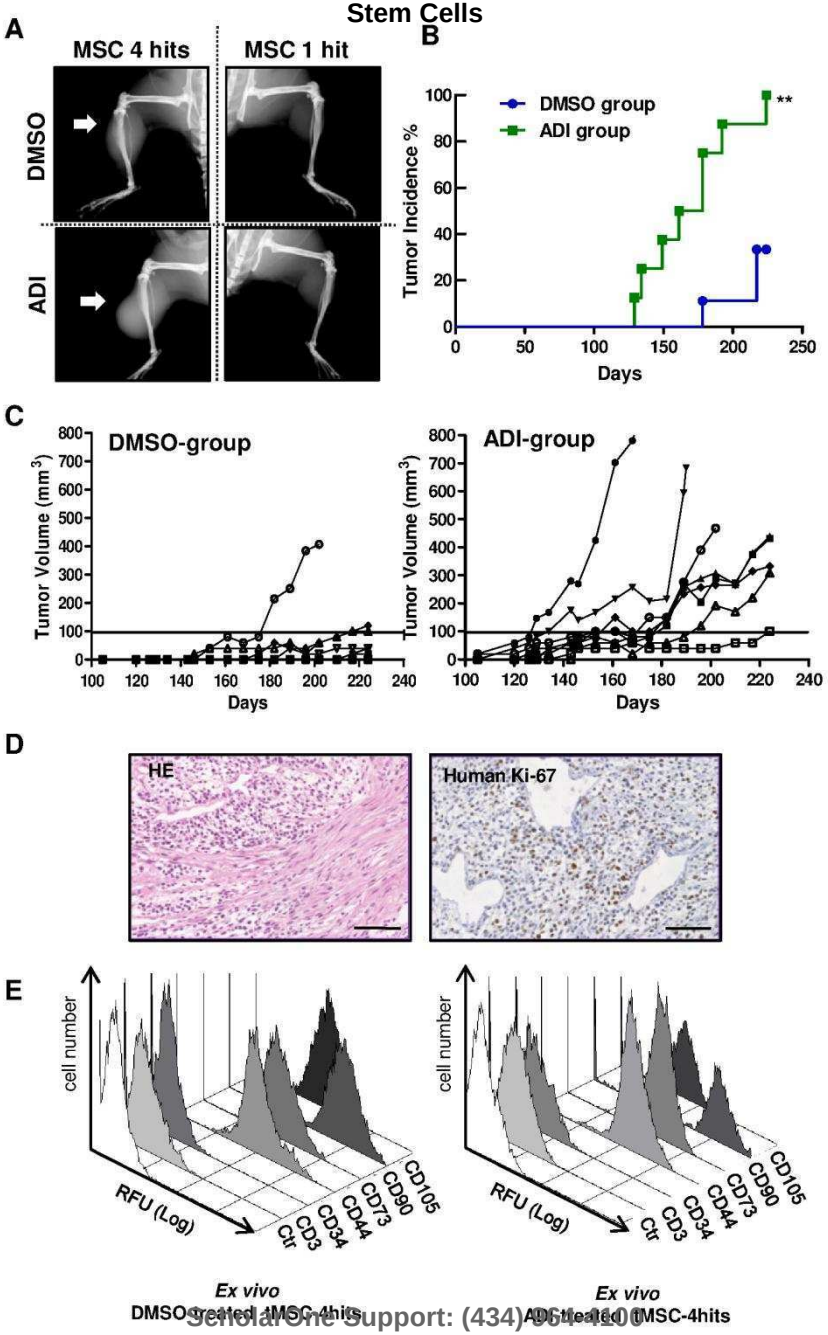




Hochane et al. Fig.5



Hochane et al. Fig.6

Low-dose pesticide mixture induces senescence in normal mesenchymal stem cells (MSC) and promotes tumorigenic phenotype in premalignant MSC.

Mazene Hochane[1,2], Valerie Trichet[3,2], Claire Pecqueur[1,2], Pierre Avril[3,2], Lisa Oliver[1,2,4], Jerome Denis[1,2], Régis Brion[3,2,4], Jérôme Amiaud[3,2], Alain Pineau[5,2], Philippe Naveilhan[6,2], Dominique Heymann[3,2,4], François M. Vallette*[1,2,7], Christophe Olivier*[1,2].

Supplementary data:

Supplementary methods

Figures S1-S10

Tables S1-S3

References (*1*-*7*)

SUPPLEMENTAL MATERIALS AND METHODS

Cell count and viability

Cells (MSC, fibroblasts and HMVEC) were seeded in their respective media and exposed to pesticide mixture for 21 days. At the end of the experiment, cells were trypsinized and counted manually by a third manipulator. MTT test was also performed. Briefly, 10 µl stock 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 50 mg/mL, Sigma) was added to each well. The samples were incubated at 37°C for 3 h, washed twice with cold PBS, and lysed with 200 µl DMSO (Sigma). Aliquots of 100 µl from each sample were transferred to a 96-well plate, and the absorbance was measured at 595 nm.

Propidium Iodide staining and BrdU incorporation

In order to evaluate the number of viable MSC, hMSC were treated for 21 days with pesticide mixture at the three different concentrations. At day 21, hMSC were trypsinized, washed with PBS and directly stained with Propidium iodide (PI) at a concentration of 500 µg/mL freshly prepared in buffer (PBS/0,2% fetal calf serum/0,1% Sodium Azide). hMSC were directly analysed by flow cytometry. Positive control for PI staining, with permeabilized cells was also assayed. The proliferation of hMSC was assayed by BrdU incorporation after 21-day treatment with pesticide mixture. BrdU was added to the culture media at day 20 and left for 24h. At day 21, cells were trypsinized, washed and resuspended in cold ethanol 70% over night. The next day, cells were washed several times to eliminate the ethanol and incubated with pepsine/HCl (40 µg/mL in HCl 0,1N) for 20 minutes. Cells were then incubated in HCl 2N at 37˚C for 1h. The acid was then neutralized with sodium Borate (0,05 M, pH 8,5) treatment at room temperature for 5 minutes. After several washes (PBS/0,1%FCS) and centrifugation, an anti-BrdU-FITC coupled was added to the cells and left for 45 minutes at RT in the dark. The cells were then washed and a solution of RNAse (200µg/mL)/PI (10µg/mL)/PBS-0,1%FCS was added and the cells were directly assayed by flow cytometry. % Cells in phase S+G2/M are analyzed.

SA-Beta-galactosidase activity

MSC, tMSC, endothelial cells and fibroblasts were seeded in 6-well plates and treated with pesticide mixture for 3 weeks before the analysis of SA-beta-galactosidase activity, distinctive of senescent cells. The analysis was done according to the manufacturer's protocol (sigma). Briefly, cells were washed twice with PBS (pH 7.2) and then fixed with (2% formaldehyde, 0.2% glutaraldehyde, 7.04 mM Na2HPO4, 1.47 mM KH2PO4, 0.137 mM NaCl and 2.68 mM KCl) for 8 min. Next, the cells were incubated in SA-β-gal staining solution (1 mg/ml X-gal, 40 mM citric acid/sodium phosphate pH 6.0, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM MgCl2) at 37°C, for 8-12h. The cells were then rinsed with PBS and observed under microscope, and SA-β-gal positive cells were counted. The experiment was performed at least three times in triplicate. A minimum of 2x103 cells were analyzed for each condition.

Measurement of reactive oxygen species (ROS)

The production of ROS was measured by the use of DCFDA probe (CM-H2DCFDA, ThermoFisher Scientific). Cells (MSC, fibroblasts and endothelial cells) were seeded in 96-well plates and treated with pesticide mixture for 21 days. Cells were incubated with the CM-H2DCFDA probe, at day 7, 14 and 21 and the fluorescence was measured at 538 nm every 3 min for 75 min. The slope of the fluorescence curve, corresponding to ROS productions, were calculated and normalized to the number of cells after Crystal Violet staining (sigma).

Immunoblot, immunofluorescence and caspase activity

Cells were lysed in RIPA lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP40, 1% Na-deoxycholate and 0.1% SDS, protease inhibitor cocktail). Protein concentration was determined by BCA protein assay (Sigma). Samples were then analyzed by SDS-PAGE and subsequently immunoblotted with different antibodies (see supplemental data for antibodies details). For the immunofluorescence assay, cells were seeded on glass slides and treated for 21 days with pesticide mixture. At day 21, cells were washed 3 times with PBS, fixed (paraformaldehyde 4%), permeabilized (PBS, 0.1 % Triton X100), blocked for unspecific binding (PBS, 3% Bovine Serum Albumin, Glycine 1mM) and incubated with the desired antibody (see antibodies table on supplemental data). The cells were then rinsed and incubated with the species specific fluorescent secondary antibody (Alexa Fluor 488 or 594, Thermo Scientific). Images were acquired with White Field Nikon Ti microscope (Nikon, Champigny-sur Marne, France).Caspase 3 activity was quantified using the fluorogenic substrate Ac-DEVD-AMC, as described in [26].

Mito Tracker staining

For staining of functional mitochondria we used Mito Tracker Red CMXRos (Life Technologies, ref. M-7512). MSC were plated on glass slides and incubated for 30 min at 37 ˚C with Mito tracker (150 nM) diluted in cell medium. The cells were then washed with PBS and fixed with 4% paraformaldehyde then mounted using ProLong Gold Antifade Mountant with DAPI (Thermofischer Scientific)

Anchorage-independent cell growth

Anchorage-independent growth was assayed by soft agarose assays. Cells were transferred to medium solution of 0.35% low melting point agarose (Sigma) and seeded in triplicate into six-well plates containing a 2-ml layer of solidified 0.6% agarose in medium at 800 cells per well. Fresh medium was added every 3 days and colonies photographed at x40 magnification after 28 days in culture.

Histology analysis

Tumor specimens were fixed in 4% buffered paraformaldehyde and after embedding in paraffin, 5 µm-thick sections were stained with an hematoxylin–eosin (HE) solution or incubated with a mouse monoclonal antibody directed against human Ki-67 protein which is a specific nuclear marker for cell proliferation. Briefly, sections were heated at

95°C within citrate buffer 1X pH 6.0 (MicromMicrotech) for 20 min, rinsed in PBS and treated with 3% H2O2 before primary antibody. Overnight incubation at 4 °C with anti-human Ki-67 (see antibody table) was performed with Tris Buffered Saline (TBS 1X pH 7.6, tween 0.05 %, triton 0.1%, bovine serum albumin 1% and 2% of goat serum). A biotinylated goat anti-mouse IgG secondary antibody (1/400, E0433, Dako) was added and detected with Streptavidin/HRP (horse radish peroxydase, Dako) complexes which were revealed by a short incubation with 3,3'- Diaminobenzidine (DAB, Dako). Nuclei were counterstained with a Gill-Haematoxylin solution. The proportion of Ki67-positive cells was calculated using ImageJ software (NIH, Bethesda, MD, USA) from counting >15000 nuclei in 3 tumor sections.

Flow cytometry analysis

For phenotypic characterization, ex vivo cultures were prepared from tumors as fllowing: tumors were dissected and tumor fragments were then dilacerated in a FCS-deprived medium before being incubated with Collagenase A (1 mg/mL) for 90 min under circular agitation. The fragments were then filtered (70 µ) and mashed and washed with MSC complete medium, centrifuged at 500 g for 10 min. the supernatants, rich with cells, were then put in culture. About 105 cells (MSC or tumor-derived cells) were suspended in 50 µL phosphate buffered saline (PBS), 0.5% BSA , 2 mM EDTA and 1 µL of one of the following antibodies: PE-conjugated mouse anti-human CD34 (clone 563), CD44 (clone 515), CD45 (clone HI30) or CD73 (clone AD2) from BD Biosciences (Le Pont de Claix, France) or PE-conjugated mouse anti-human CD90 (clone5E10) or CD105 (clone 43A3) from BioLegend through Ozyme (Paris, France). After washing in PBS, cells were analyzed with flow cytometer (Beckman Coulter FC500).

FIGURE S1

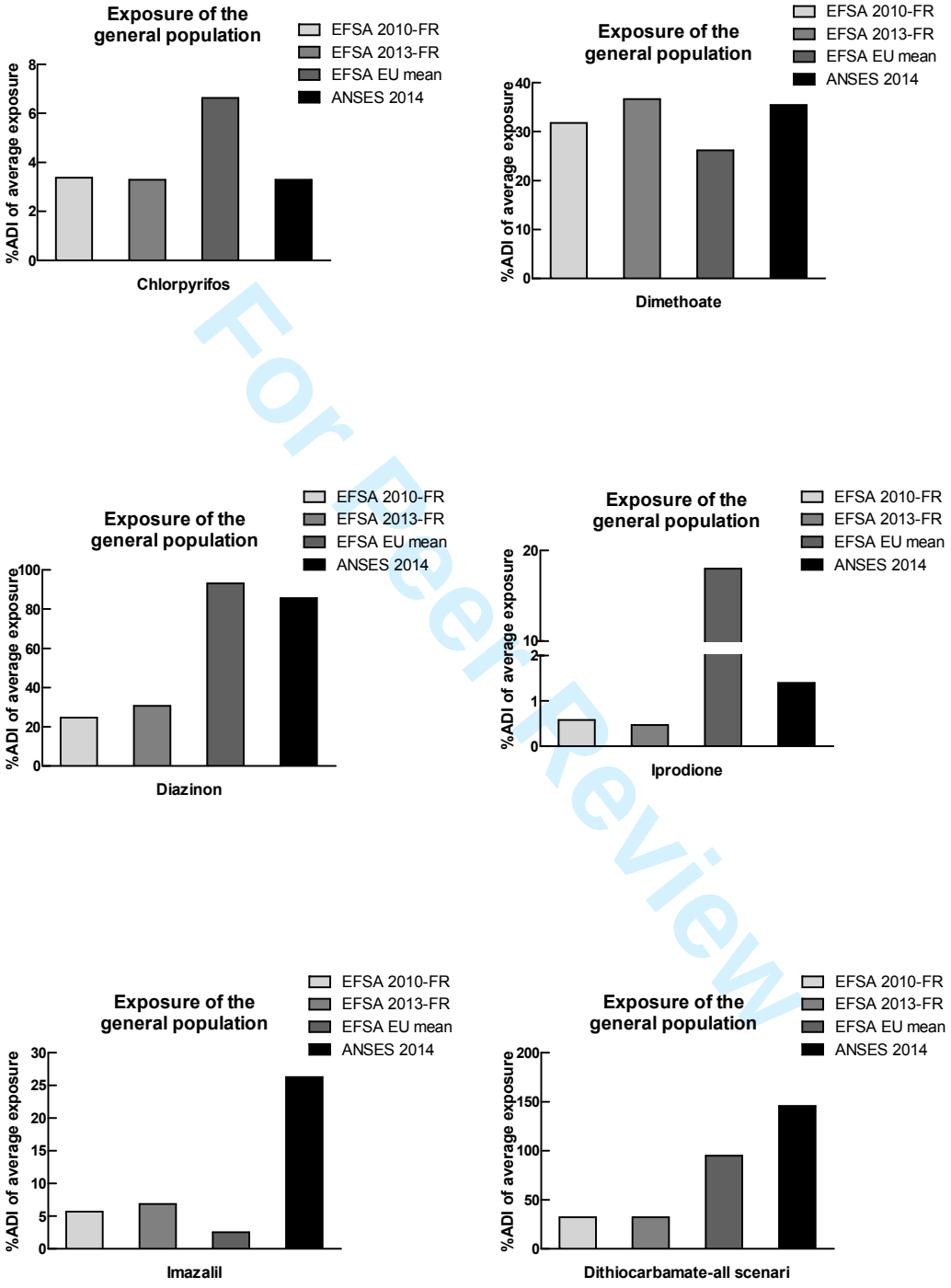


Fig. S1.

Mean quantities of pesticides residues found in French and European food as reported by EFSA 2010, EFSA 2013 and ANSES 2014. Values are presented for each pesticide as %

of its ADI. The mean quantities of our 7 pesticides (Chlorpyrifos, Diazinon, Dimethoate, Iprodione, Imazalil, Maneb and Mancozeb) reported by three major studies (EFSA,2010[1] and 2013[2] for French and European general populations) and ANSES, 2014[3] (*AgenceNationale de SécuritéSanitaire de l'Alimentation*, for French population). In these reports, the values of the mean chronic exposures were calculated for each pesticide as percents of its Acceptable Daily Intake (ADI). For maneb and mancozeb, results were depicted as Dithiocarbamate-all scenari because the method of detection makes no specific distinction between these two compounds. In our study, we used the values reported by EFSA,2010.

1- http://www.eurosfaire.prd.fr/7pc/documents/1279027807_rapport_efsa_15_juin_2010.pdf

2-http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/4038.pdf

3- https://www.anses.fr/fr/system/files/AUT2013sa0138.pdf

FIGURE S2

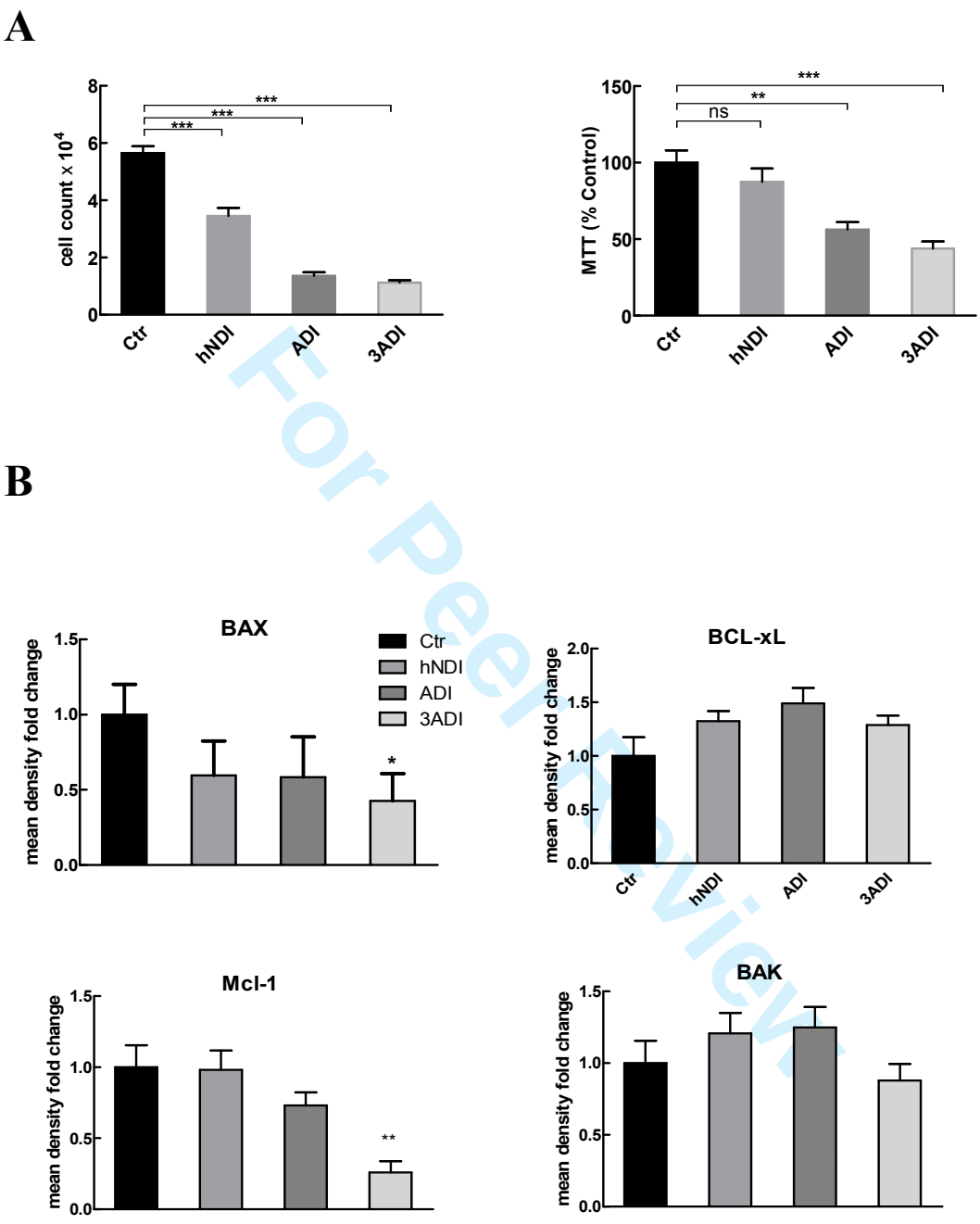


Fig. S2

(**A**) MSC proliferation in the absence or in the presence of pesticide mixture for 21 days. MSC were seeded at 2000 cells/cm^2 and treated with DMSO 1/1000 v/v (Ctr) or pesticides mixture for 21 days. Cell viability was analyzed by MTT assay staining, and growth by cell counting at the end of the experiment, n=5, * p< 0.05, ***p*<0.01 *vs* Ctr. (**B**) Semi-quantification of the expression of pro and anti-apoptotic proteins in MSC after 21-day exposure to pesticide mixture (n=3).

FIGURE S3

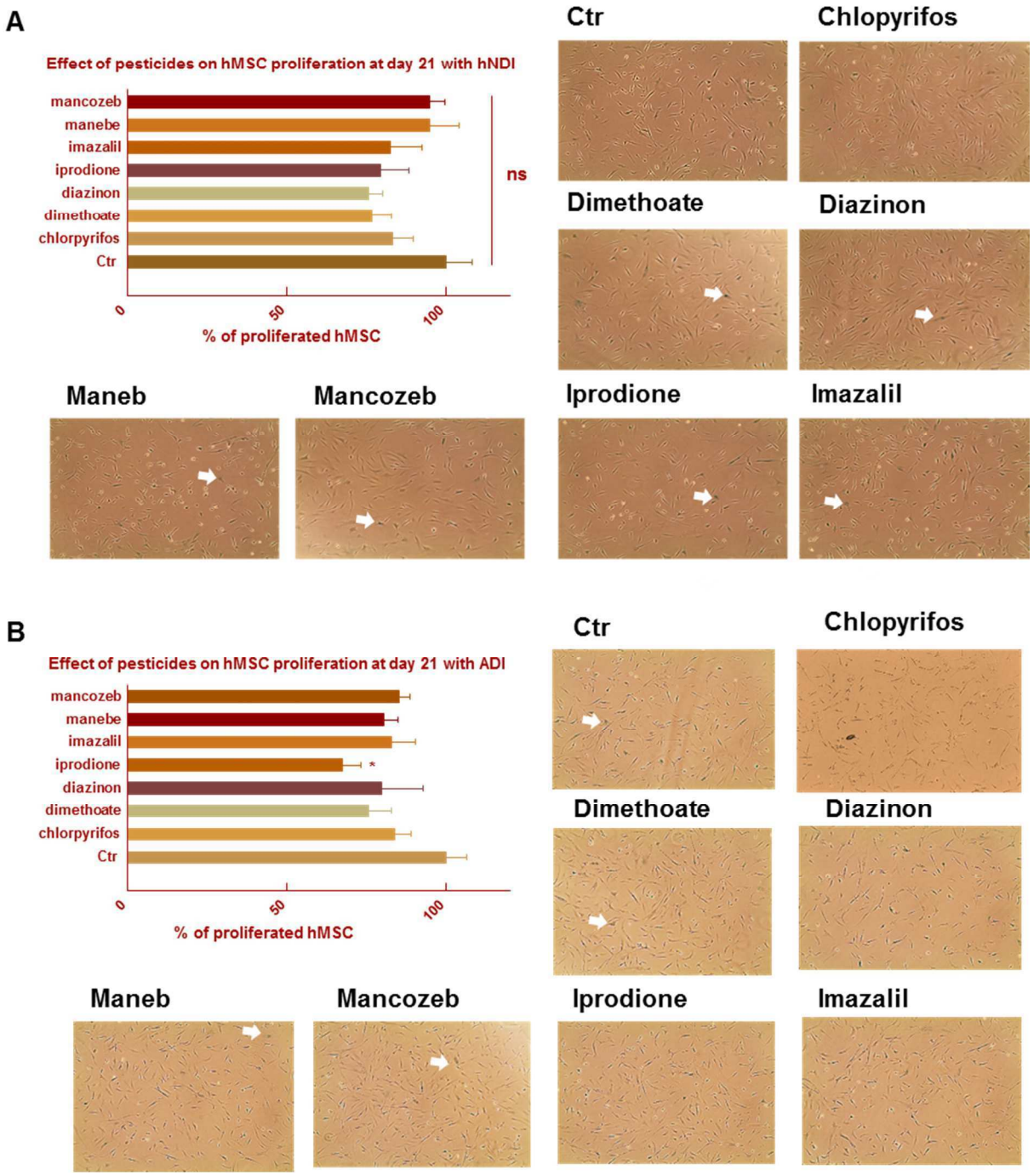


Fig. S3
Individual pesticides used in the pesticide mixture have no effect on MSC viability or senescence at hNDI and ADI dose after 21-day treatment. MSC were treated with Chlorpyrifos, Diazinon, Dimethoate, Iprodione, Imazalil, Maneb and Mancozebindividually for 21 days at hNDI (**A**) and ADI (**B**) doses. MSC proliferation and senescence were then assessed by MTT and β-Galactosidase staining (*p<0.05, n=3).

FIGURE S4

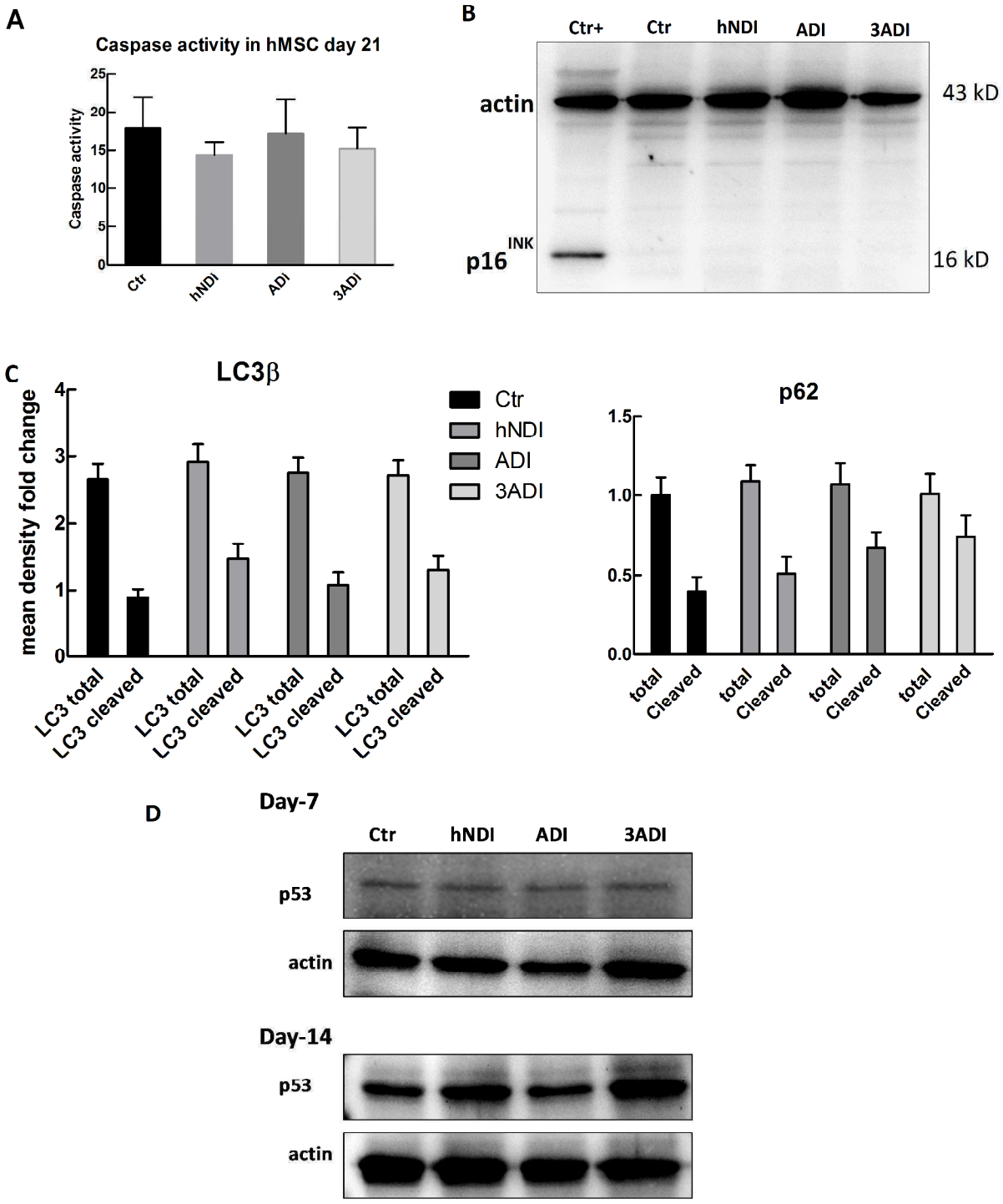


Fig.S4

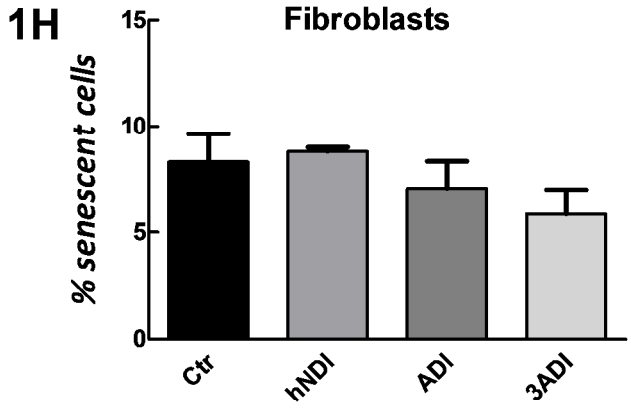
MSC were treated with pesticide mixture for 21 days. (**A**) Caspase-3 activity was analyzed in protein extracts using the fluorogenic substrate Ac-DEVD-AMC (n=3) (**B**) Immunoblot analysis of $p16^{INK4a}$ in MSC shows no expression at day 21 (n=3). The positive control (Ctr+) consists of senescent HMVEC cultivated for 21 days after

irradiation at 15 gy. (**C**) Semi-quantification of LC3ß cleavage and p62/SQSTM1 degradation after 21-day exposure to pesticide mixture (n=3). (**D**) p53 expression, analyzed by immunoblot at day 7 and 14 after pesticide mixture-treatment (n=3).

FIGURE S5

Quantification of β-gal staining for figure 1G (endothelial cells): β-gal staining was completely negative

Quantification of β-gal staining for figure 1H



Quantification of β-gal staining for figures 2B :

2B: The treatment with pifithrin-α totally abolished the senescence in MSC, β-gal staining was completely negative

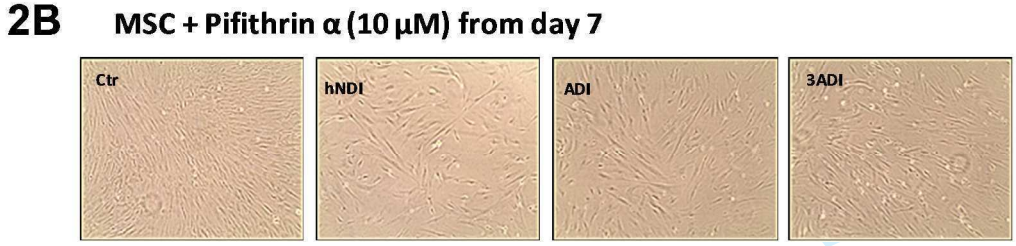


Fig.S5,
Upper panel, quantification of beta-gal staining in endothelial cells (HMVEC) and human fibroblasts (for figure 1A and 1B) after 21-day pesticide mixture exposure.
Lower panel, quantification of beta-gal staining in MSC after treatment with pifithrin-α (10 µM from day 7) during the 21-day exposure to pesticide mixture.

FIGURE S6

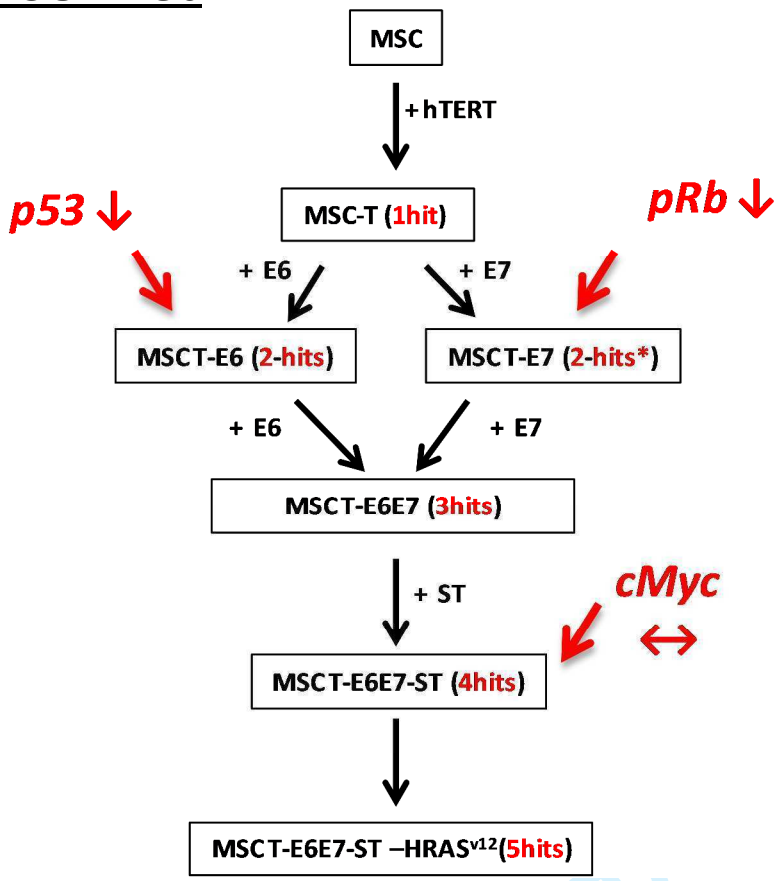


Fig.S6

Transformation of MSC, according to Funes JM*, et al. 2007*

MSC were modified and transformed by a stepwise retroviral transduction as follows
-The catalytic subunit of human telomerase (hTERT) (tMSC-1hit), prolonging lifespan
-tMSC- 2hits, + HPV-16 E6 which abrogate the functions of p53 or MSC-2hits* + HPV-16 E7 which abrogate pRB family members
-tMSC- 3hits, + HPV-16 E6/E7
-tMSC- 4hits, + SV40 small T antigen (ST), ST inactivates protein phosphatase 2A, resulting in c-Myc stabilization
-tMSC- 5hits, + Oncogenic allele of H-Ras (H-Ras^{V12}), H-Ras^{V12} provides acquisition of a constitutive mitogenic signal.

FIGURE S7

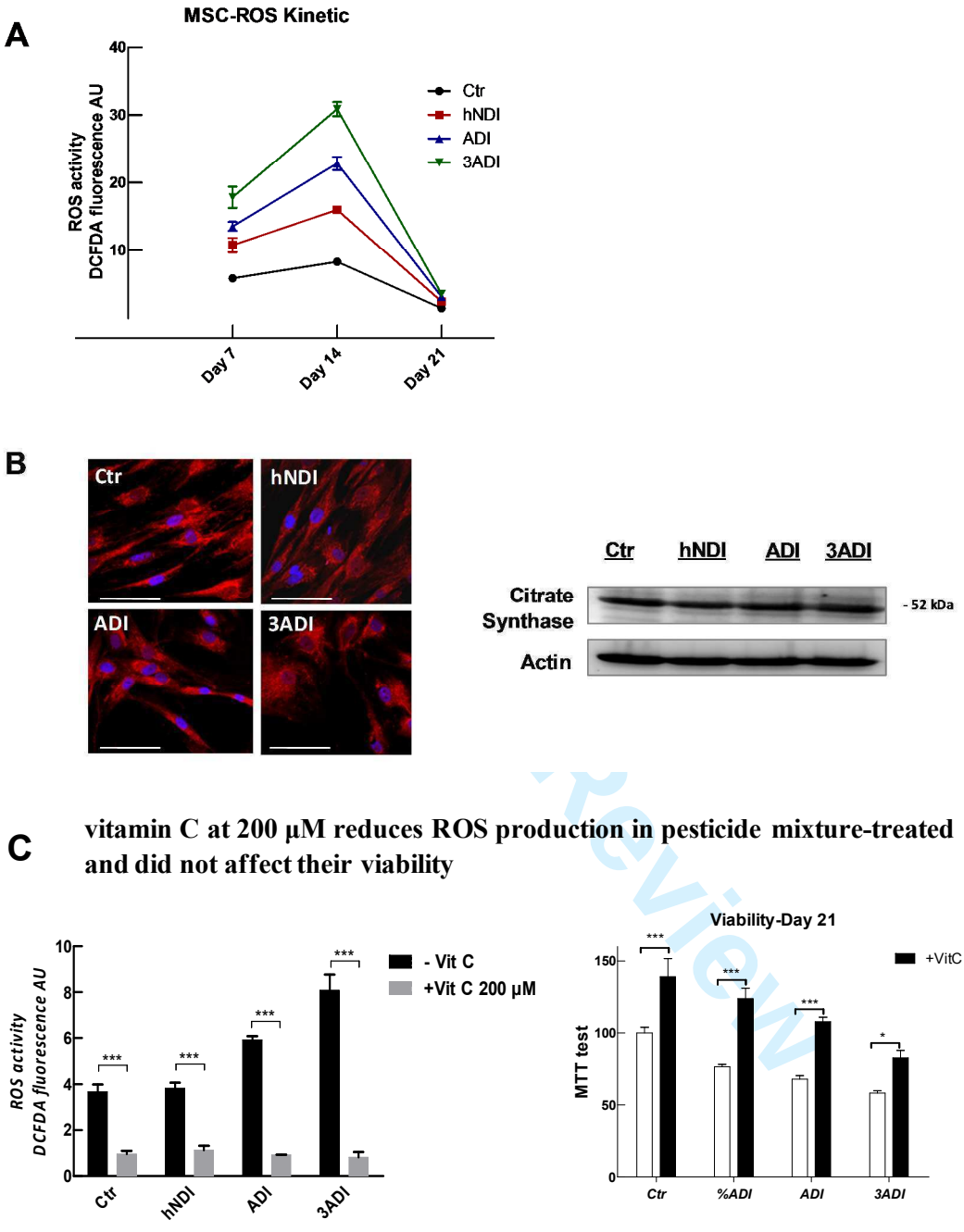


Fig.S7

Pesticide mixture induces a dose-dependent ROS production in MSC at day 7 and day 14 but does not decrease functional mitochondrial number. (**A**) MSC were treated with pesticide mixture and ROS production was measured using CMH_2-DCFDA. For each condition, results are normalized to the cell content after Crystal violet staining. (*p<0.05, ***p<0.001, n=3).(**B**)Detection of functional mitochondrial network in MSC was performed using Mito Tracker Red (150 nM) at day 21. Citrate-synthase expression

was analyzed in MSC after exposure to pesticide mixture for 21 days by immunoblot. (**C**) MSC were treated with vitamin C (200 µM) in the presence or in the absence of pesticide mixture during 21days. Cell viability was measured at day 21 using MTT assay (**p<0.05, ***p<0.01, n=3)

FIGURE S8

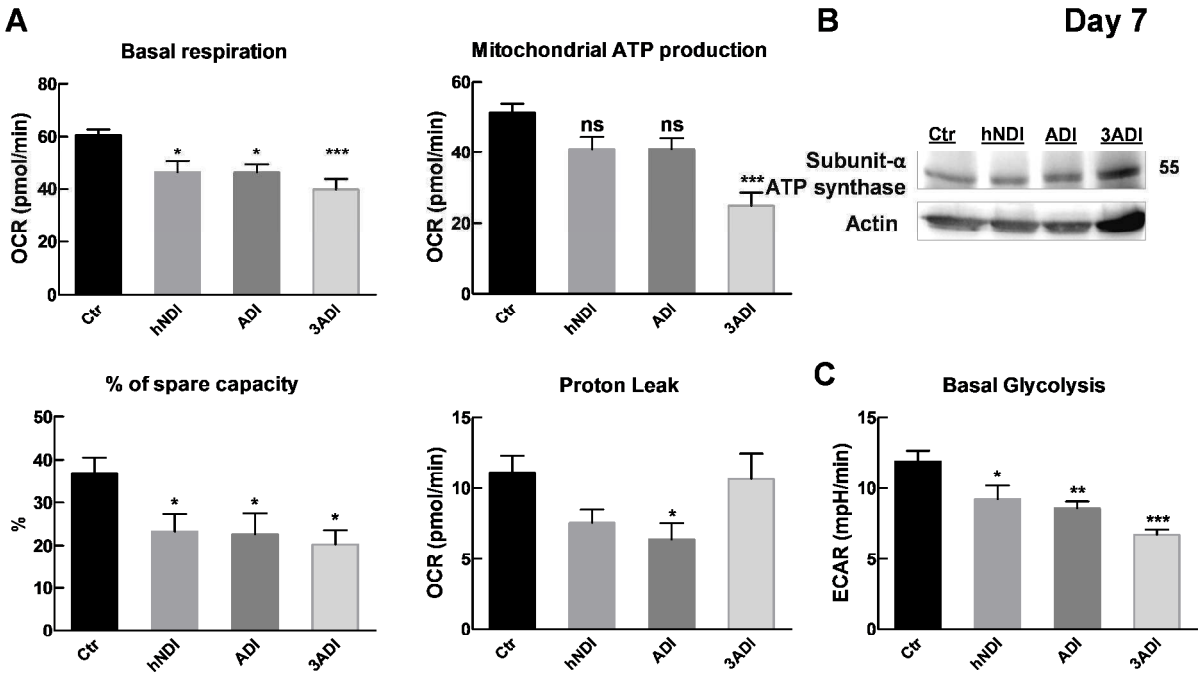


Fig.S8

A, Mitochondrial respiration was assessed using the Seahorse XF24 Flux Analyzer. After 7 days of exposure to pesticide mixture, cells ($2x10^4$) were seeded in 24-well XF plate and the oxygen consumption rate (OCR) was measured before drug addition (basal respiration), mitochondrial ATP production was estimated after oligomycin treatment, proton leak was the result of the excess OCR between basal OCR and values after oligomycin addition. The % spare respiratory capacity was calculated after CCCP treatment. **B**, The expression of ATP synthase (subunit-α) was analyzed by immunoblot at day 7. **C**, Basal glycolysis was estimated from the ExtraCellular Acidification Rate (ECAR) before addition of drugs. (n=5, *p<0.05, **p<0.01).

Mitochondrial respiration was significantly affected after 7-day pesticide mixture treatment with a reduction in cellular basal respiration regardless of the dose. This reduction was not associated with less mitochondrial ATP generation in presence of hNDI and ADI but decreased by 50% with 3ADI (oxygen consumption rate OCR=51.37 ± 2.49 pmol/min in control *versus* 24.9 ± 3.67 with 3ADI, p<0.01). The reduction in basal respiration with the preservation of constant ATP production with hNDI and ADI doses may be due to the exhaustion of the metabolic resources and the limitation of proton leak. In fact, a major part of the spare metabolic capacities, which is the extra capacity available in cells to produce energy in response to increased stress, was in use as about 20% ± 4 the spare mitochondrial capacity was preserved versus an average of 35% ± 3 in control cells (p<0.05). No effect on proton leak was seen at day-7 and the expression of ATP synthase was not modified. Pesticides-exposed cells showed less basal glycolytic ability than control cells in a dose-dependent manner.

FIGURE S9

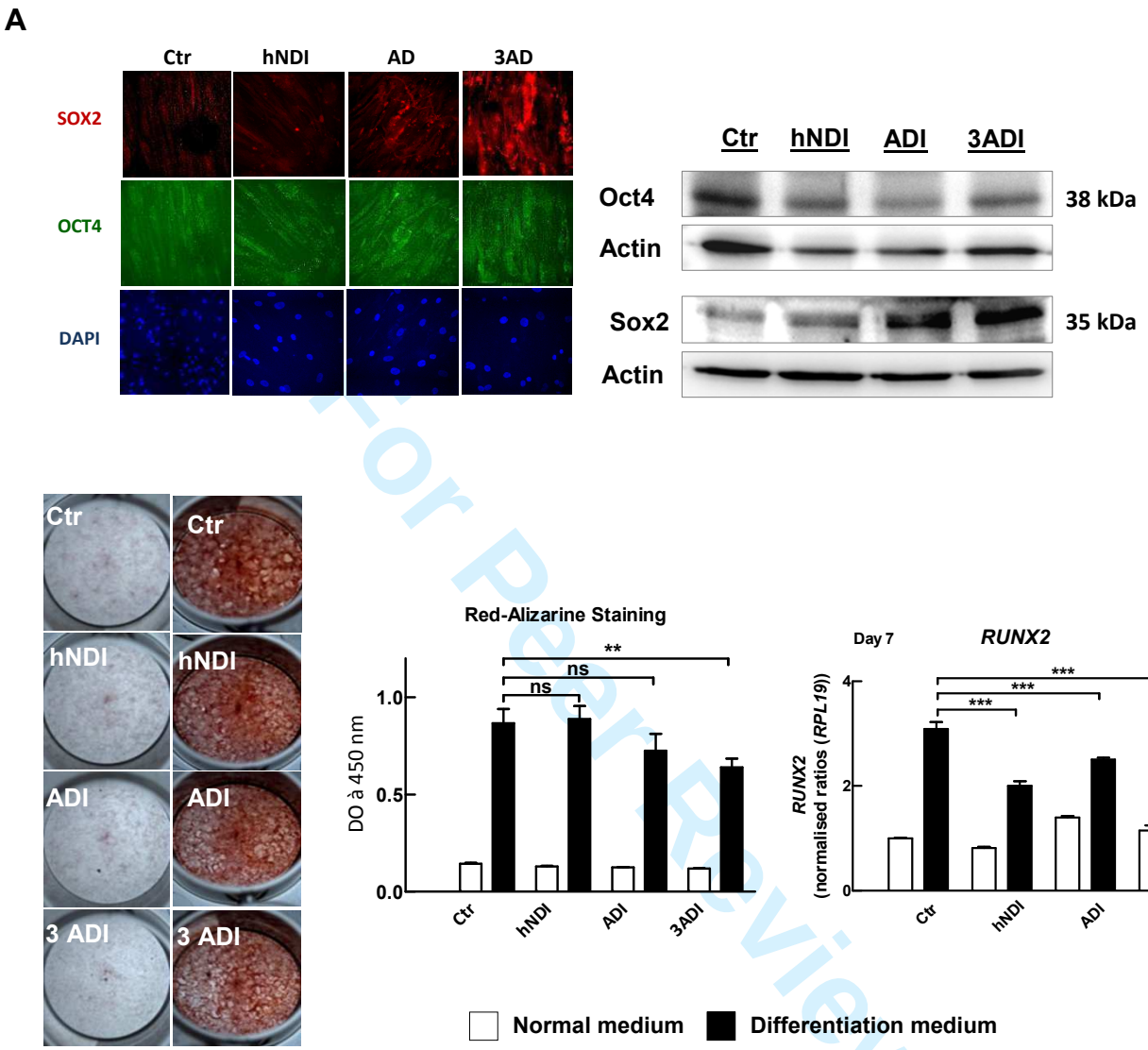


Fig.S9

(**A**) Expression of stemness markers Sox2 and Oct4 in MSC was evaluated by immune flurescence(bars 200µm) and immunoblt analysis after 21-day exposure to pesticide mixture(n=3). (**B**)MSC were seeded in 6-well plates and treated for 3 weeks with pesticide mixture in the presence or absence of osteogenic differentiation media, red-alizarin staining was performed and representative photos were taken for each condition. Red alizarin was then dissolved and optical density measured at 450 nm (n=3, $**p<0.01$). Total RNA was extracted from the cultures and RUNX2 transcripts were quantified by RT-qPCR at day 7 after osteogenic induction. Results are normalized to domestic gene RLP19 mRNA (n=3, $***p<0.001$). (**C**) MSC were treated with pesticide mixture in the presence of osteogenic differentiation medium. Total RNA was extracted and *ALP* transcripts were quantified by RT-qPCR at day 7, 14 and 21 of MSC differentiation. Results are normalized to *RLP-19* mRNA used as a domestic gene (ns = not significant, n=3).

FIGURE S10

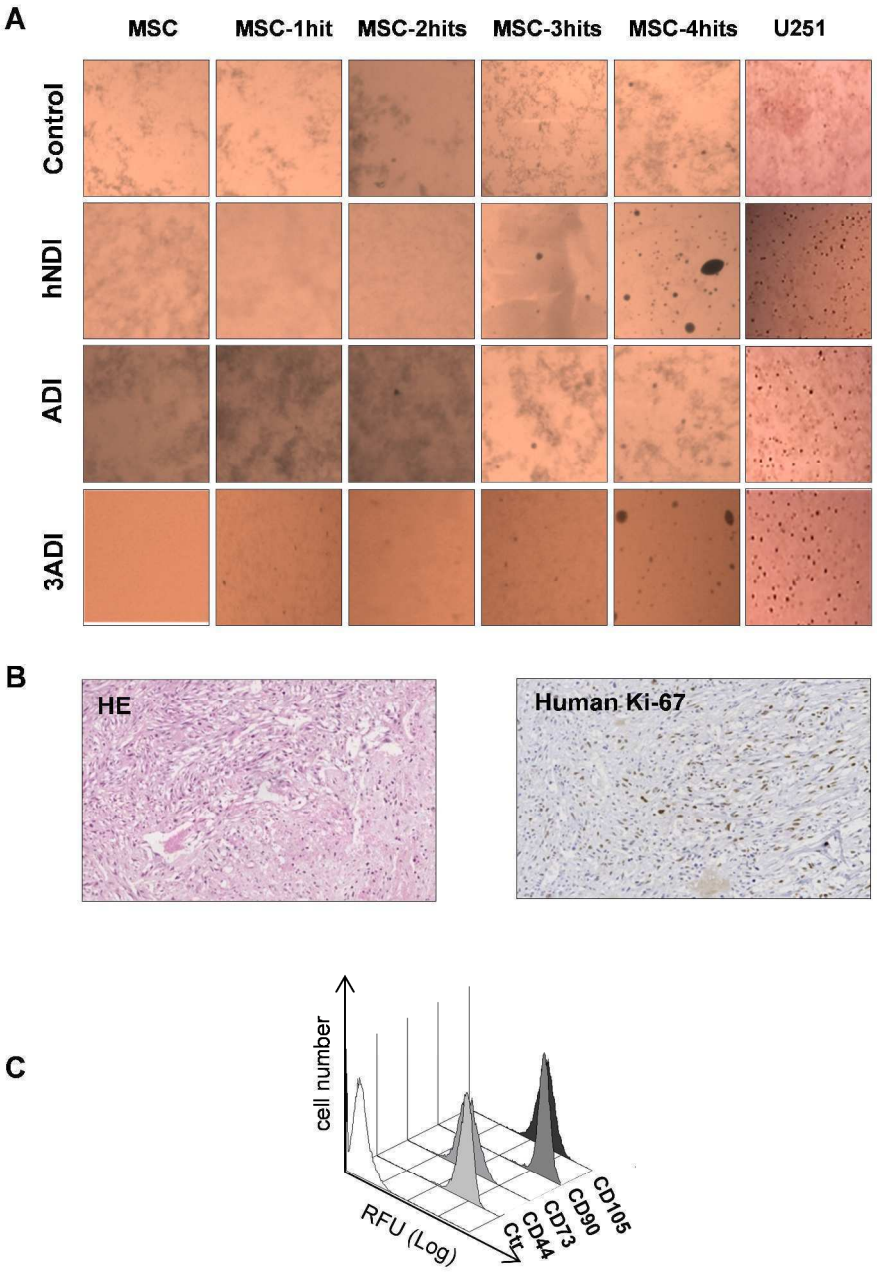


Fig.S10

(**A**), naïve MSC and modified tMSC were treated for 21 days with pesticide mixture then seeded (800 cells) in soft agar. Colony formation was analyzed 28 days later (n=3), glioblastoma cell-line U251 were assessed similarly (positive control).(**B**)Histological analysis of tumour obtained from DMSO-group (HE staining) and proliferating human cells specifically detected with anti-human Ki-67. (**C**)Flow cytometry analysis of stemness markers in parental tMSC-4hits cells before any treatment.

TABLE S1

Protein	Host species	MW (kDa)	supplier	Reference	Dilution Wb	Dilution IF
Actin	mouse	43	Millipore	MAB1501R	1/2000	-
ATP synthase	mouse	60	Invitrogen	429240	1/500	-
Bak	rabbit	24	BD	556396	1/1000	
Bax	rabbit	21	Cell signaling	2772	1/1000	
Bcl-Xl	Mouse	26	BD	610747	1/1000	
Catalase	rabbit	60	Abcam	ab16731	1/2000	
Citrate synthase	rabbit	52	Novus	NBP2-	1/1000	-
GPX-1	rabbit		Abcam	ab16798	1/1000	-
GPX-2	rabbit	22	Abcam	ab137431	1/1000	-
Ki67 (clone	mouse	345, 395	Dako	M7240		1/100
LC3-β	rabbit	16, 18	Sigma	L7543	1/1000	-
Mcl-1	rabbit	31, 43	Santa cruz	Sc-819	1/500	
OCT4	rabbit	44	Millipore	MAB4401	1/1000	1/200
$P16^{INKa4}$	mouse	16	BD	554079	1/1000	
$P21^{waf1}$	mouse	21	BD	556430	1/1000	-
P53	mouse	53	BD	554294	1/1000	-
P62 (SQSTM1)	rabbit	60	Cell signal	5114	1/500	-
SOD-1	mouse	32	BD	556360	1/1000	-
SOD-2	rabbit	25	Abcam	ab16956	1/1000	-
Sox-2	rabbit	35	Active Motif	39823,39824	1/2000	1/500

Table S1.
Table S1 lists the antigens, working concentrations, references, suppliers and origins of the antibodies used in the study

TABLE S2

Pesticide	Molar mass	ADI (mg/kg b.w./d)	Average exposure according to EFSA 2010	CellsExposure concentrations (µmol/l)		
	(g/mol)	EFSA 2010	hNDI (in % ADI)	hNDI	ADI	3 ADI
Chlorpyriphos	350.59	0.01	3.30	0.01	0.3	0.9
Dimethoate	229.26	0.001	24.67	0.01	0.1	0.3
Diazinon	304.35	0.0002	36.67	0.003	0.01	0.03
Iprodion	330.17	0.06	0.47	0.01	2.2	6.6
Imazalil	297.18	0.025	6.87	0.07	1.0	3
Maneb	265.3	0.05	32.07	0.73	2.3	6.9
Mancozeb	266.51	0.05	32.07	0.72	2.3	6.9

Table. S2.

Table **S2**summarizes the doses of pesticides used: the three doses of pesticides in the mixture were calculated as the concentration of the pesticide unchanged that may be ingested by a person of 60 Kg body weight. For each molecule, the doses were extrapolated from its Acceptable Daily Intake (ADI), the highest Nutritional Daily Intake (hNDI) being the average of the highest estimated amounts of a pesticide to which the all-age French population is exposed according to the European Food Safety Agency(*5*) report (2010), and 3 times the ADI (3ADI). It is noteworthy that hNDI doses of these pesticides varied between 0.6 to a maximum of 32.4 the percentage of ADI values. Throughout the paper, the nomenclatures: hNDI, ADI and 3ADI indicate the mixture doses extrapolated of these three values.

TABLE S3

Cells	*Hits*	*Ctr*	*hNDI*	*ADI*	*3ADI*
U251	0	393	1298	784	1155
MSC	0	0	0	0	0
T	1	0	1	2	0
T-E6	2*	0	4	6	6
T-E7	2	4	8	6	6
T-E6E7	3	91	172	67	126
T-E6E7-ST	4	77	781	192	612

Table. S3.

Number of colony formed in soft agar at day 28 in normal MSC and different tMSC following 21-day exposure to pesticide mixture.Cells were pretreated for 21 days with pesticide mixture then 800 cells were seeded in soft agar for 28 days, photos were then taken and colonies were counted.

Additional references

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6. C. Gratas, Q. Sery, M. Rabe, L. Oliver, F. M. Vallette, Bak and Mcl-1 are essential for Temozolomide induced cell death in human glioma. *Oncotarget***5**, 2428-2435 (2014).
7. K. J. Livak, T. D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods***25**, 402-408 (2001).