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Low-Dose Pesticide Mixture Induces Senescence in Normal Mesenchymal Stem Cells (MSC) and Promotes Tumorigenic Phenotype in Premalignant MSC

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Key Words. mesenchymal stem cells • pesticides • pesticide mixture • senescence • tumorigenesis

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. STEM CELLS *2017;35:800–811*

SIGNIFICANCE STATEMENT

The environmental exposure to pesticides is recognized as a potential threat to human health. However, little is known on the effect of pesticides on stem cells. Here we show that a pesticide mixture, at doses commonly found in western diet, promotes stress-related senescence in normal MSC and tumorigenesis in premalignant MSC. Our results provide a new insight in the effect of pesticides on the acceleration of stem cell ageing and possibly on cancer.

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 nonoccupational 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 United States, 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, European Food Safety Agency (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

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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 favor the onset of cancer in predisposed MSC.

Materials 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). 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 no. 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-minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, and fetal calf serum (FCS) (#10270) were from ThermoFisher Scientific (Villebon-sur-Yvette, France). Transformed MSC (tMSC) 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 (Endothelial Basic Medium (EBM)-2) were from Lonza (Viviers, Belgium). The references of the antibodies used in this study are listed in Supporting Information 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 (3–220 times less than the ADIs). The next doses are the international 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 and 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 percent 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 l 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 Dimethyl-sulfoxyde (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 3 days.

Cells Culture and Treatment

Except for the experiments on MSC differentiation, MSC were cultured at 2000 cells/cm^2 in alpha-MEM modified with ribonucleosides and deoxyribonucleosides and supplemented with FCS (10%) and 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.2 ng/ml of Fibroblast Growth Factor-2 (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 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). 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 pifithrin-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 2×10^4 cells/well and left overnight without the pesticide mixture to adhere before the analysis. Cells were equilibrated for 1 hour 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), Carbonyl cyanide m-chlorophenyl hydrazone (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 oxygen consumption rate (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 (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/cm^2 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 (nondifferentiation 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 GTA GCTGTACTCATCTTCATAGGC and RLP19 forward primer GCTCTT TCCTTTCGCTGCT 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 four chambers glass slides (Labtek, Dominique Dutscher, Brumath, France) at 2×10^4 cells/cm^2 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 High-content Screening (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. At 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 500–1500 mm^3. Tumor volume was calculated according to the following formula: $(l^2 \times L)/2$ 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 $p < .05$ were considered statistically significant.

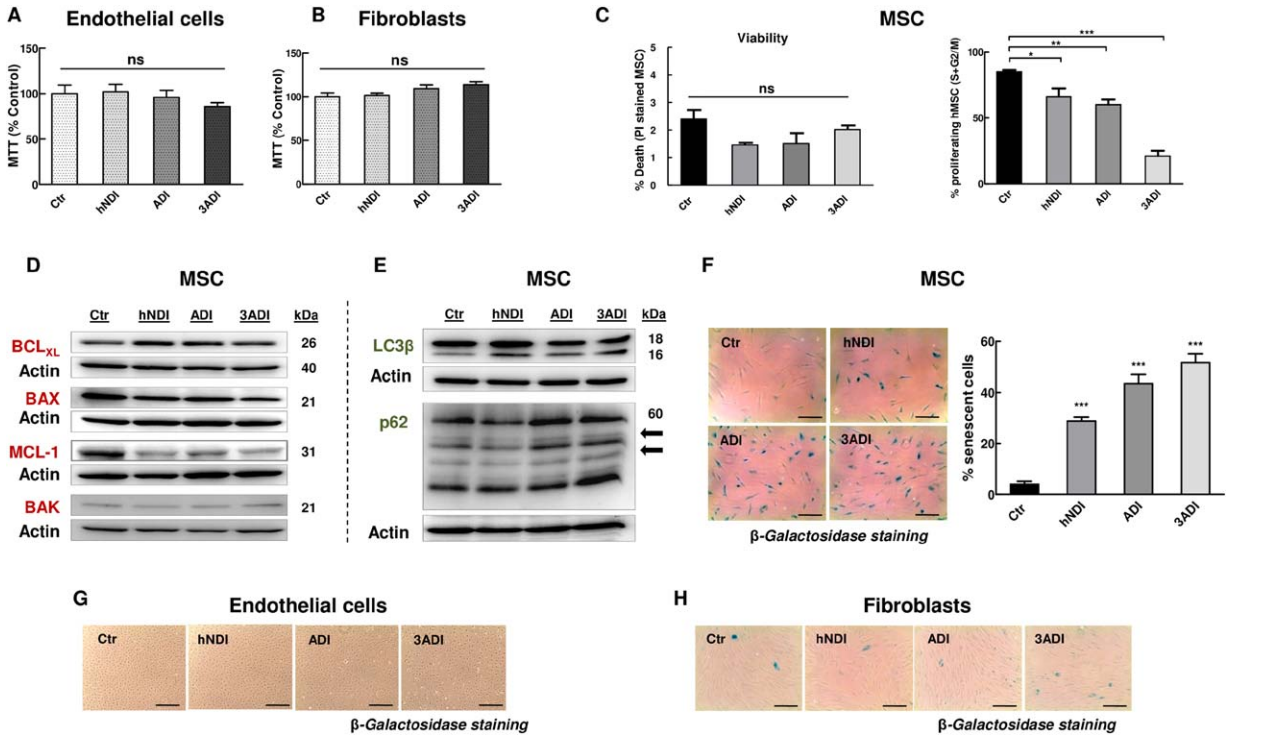

Figure 1. Effect of pesticide mixture on fibroblasts, endothelial cells, and MSC. **(A)**: Endothelial cells were treated or not with pesticide mixture for 21 days and cell growth was analyzed by MTT at day 21 ($n = 3$). **(B)**: Fibroblasts were exposed to pesticide mixture for 21 days and assayed as 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 PI staining, and proliferation by BrdU incorporation at the end of the experiment, $n = 3$, * $p < .05$, ** $p < .01$ versus 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. ($n = 3$, $p < .01$ vs. Ctr) (Bars of 200 μm). **(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 ($n = 3$). (Bars of 200 μm). Abbreviations: ADI, acceptable daily intake; hNDI, high nutritional daily intake; MSC, mesenchymal stem cells; MTT, 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide; PI, propidium iodide.

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 Supporting Information - Supplemental Materials and Methods.

Results

Specific Induction of Senescence in MSC by an Environmental Combination of Pesticides

In order to mimic the heterogenic human exposure, seven 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] (Supporting Information Fig. S1 and TableS2). 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, HMVEC and MSC. As shown in Fig. 1A and 1B, 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 (Supporting Information Fig. S2A) without affecting their viability, evaluated by propidium iodide staining (Fig. 1C) and MTT assay (Supporting Information Fig. S2A). Of note, individual exposure of MSC to each pesticide did not significantly alter cell proliferation with doses of hNDI and ADI (Supporting Information 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 (Supporting Information Fig. S4A). The expression of some proteins of the BCL2-family was deregulated (i.e., BCL2 associated X protein (BAX) and myeloid cell leukemia-1 (MCL-1) expressions were decreased and BCL$_{XL}$ was increased) which suggested an increased resistance to apoptosis (Fig. 1D and Supporting Information 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 Supporting Information 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 1H and Supporting Information 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] (Supporting Information Fig. S6). In naïve MSC, the pesticides induced an upregulation of $p21^{waf1}$ but not in p53 expression at day 21 or earlier (Fig. 2A and Supporting Information Fig. S4D), while $p16^{INK4a}$ was not detected by Western blot in these cells under our conditions (Supporting Information Fig. S4B). In addition, the inhibition of the transcriptional activity of p53 by pifithrin-α (10 μM from day 7) (Supporting Information 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, $p21^{waf1}$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 analyze the effect of the pesticide combination, the generation of ROS was analyzed at day 7, 14 (Supporting Information 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 (Supporting Information 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 (Supporting Information 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 (Supporting Information 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 (Supporting Information 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, $p < .05$). 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

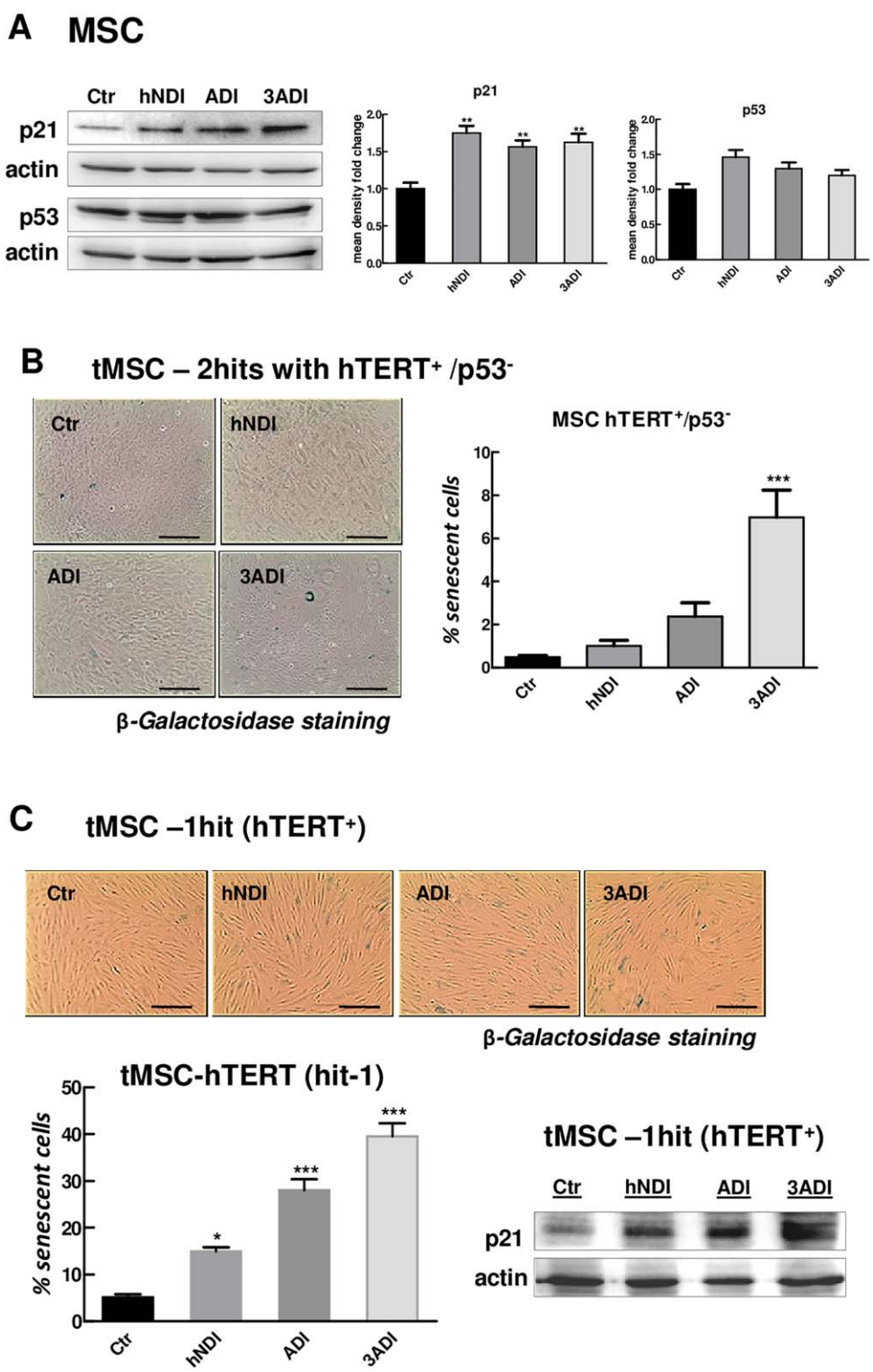

Figure 2. Pesticide mixtures induce p53-dependant but not telomere-dependant senescence in MSC. **(A)**: Immunoblotting and semi-quantification of $p21^{waf1}$ and p53 in MSC at day 21 after exposure to pesticide mixture ($n = 3$). **(B)**: β-gal staining and quantification in transformed tMSC-2hits ($hTERT^{+}/p53^{-}$) 21 days after pesticide exposure ($n = 3$) (bars of 200 μm). **(C)**: β-gal staining in tMSC-1hit ($hTERT^{+}$) in presence of pesticides at day 21 (bars of 200 μm). The percentages 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 ($n = 3$, $*p < .05$, $**p < .01$,$***$ $p < .001$). Abbreviations: ADI, acceptable daily intake; hNDI, high nutritional daily intake; hTERT, human telomerase MSC, mesenchymal stem cells; MTT, 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide; tMSC transformed mesenchymal stem cells; PI, propidium iodide.

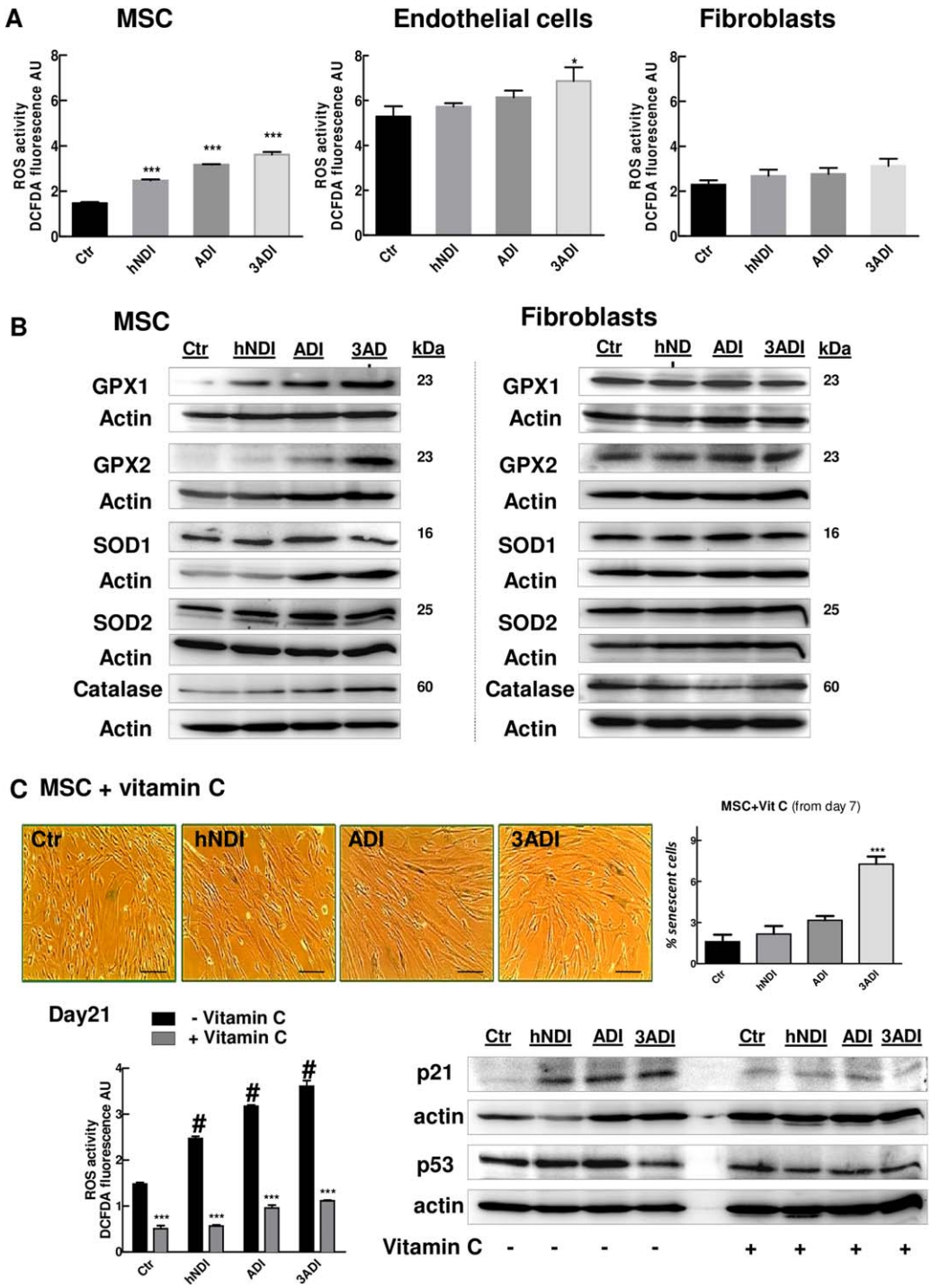

Figure 3. Pesticide mixture enhances ROS production specifically in MSC and oxidative stress reversion abrogates pesticide-induced senescence in MSC. **(A)**: ROS production was analyzed in MSC, fibroblasts, and endothelial cells using CMH_2-DCFDA at day 21 ($n = 3$, $^*p < .05$, $^{**}p < .01$). **(B)**: 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, bars of 200 μm). ROS activity in MSC in presence of vitamin C at day 21 ($^{***}p < .01$ vs. vitamin C treatment, $^{\#}$ $p < .05$ Ctr versus pesticide-treatment, $n = 3$), expression of $p21^{WAF1}$ and p53 under the same conditions ($n = 3$). Abbreviations: ADI, acceptable daily intake; CMH2-DCFDA, Chloromethyl-2′,7′–dichlorofluorescin diacetate; GPX1, glutathione peroxidase hNDI, high nutritional daily intake; hTERT, human telomerase MSC, mesenchymal stem cells; MTT, 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; SOD2, superoxide dismutase; tMSC transformed mesenchymal stem cells; PI, propidium iodide.

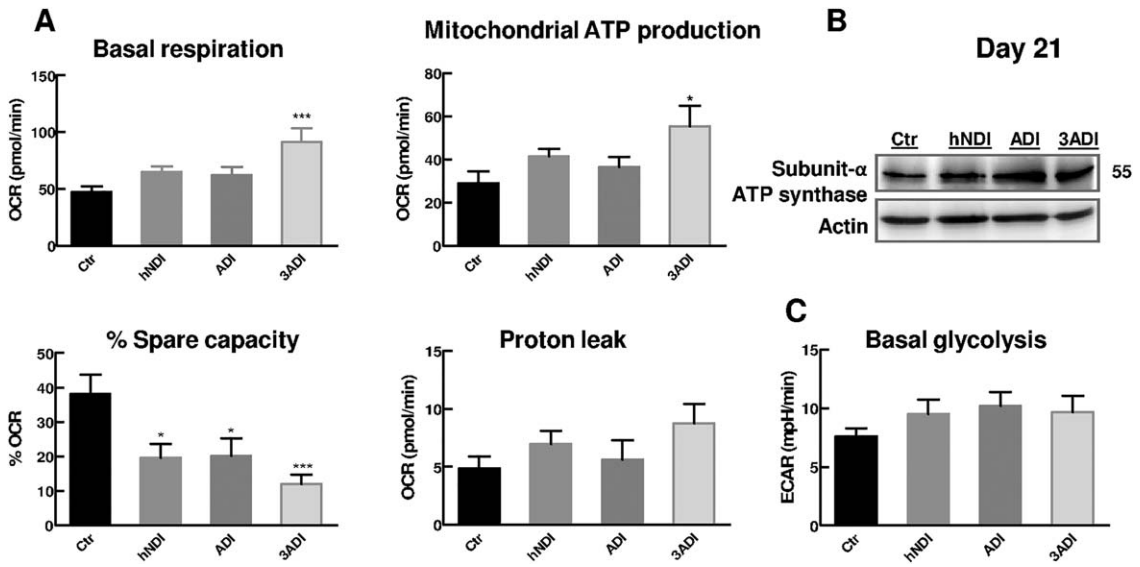

Figure 4. Pesticide mixture causes respiratory changes in mesenchymal stem cells. **(A)**: Mitochondrial respiration was assessed using the Seahorse XF24 Flux Analyzer. After 21 days of exposure to pesticide mixture, cells (2×10^4) were seeded in 24-well XF plate and the OCR was measured before drug 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 percent 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 ECAR before addition of drugs. ($n = 3$, $*p < .05$, $**p < .01$, $***p < .001$). Abbreviations: ADI, acceptable daily intake; ECAR, extracellular acidification rate; hNDI, high nutritional daily intake; OCR, oxygen consumption rate.

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 (Supporting Information 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 (Supporting Information 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 (Supporting Information 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 (Supporting Information 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 pretreatment with pesticide mixture enhanced the colony formation after 28 days in the soft agar assay (Supporting Information 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 three of nine mice showed tumour growth in DMSO-group all over the experiment while all the mice in ADI-group developed tumours ($p = .011$). 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 myxoid 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 ($>13\% \pm 6$) 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, Supporting Information 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.

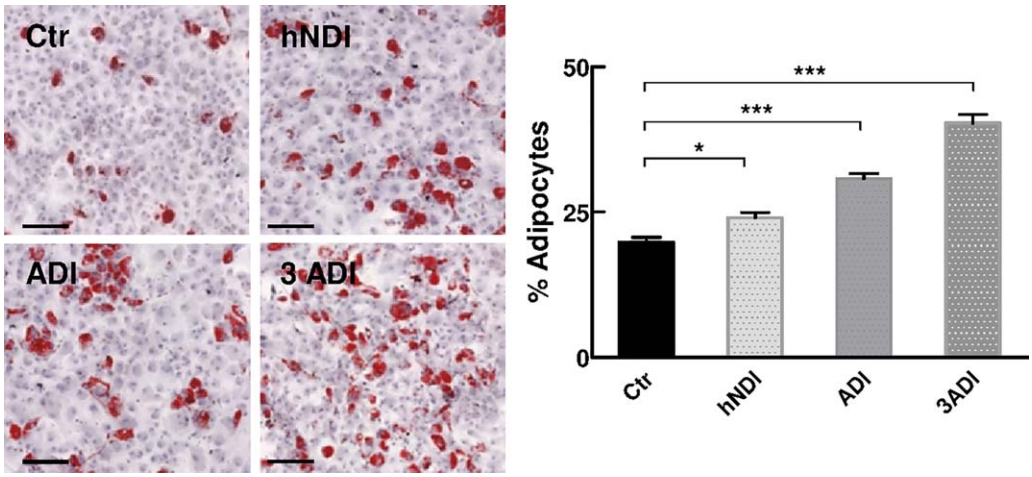

Figure 5. Pesticide mixture promotes adipogenic transformation in mesenchymal stem cells (MSC). After 1-week treatment with pesticide mixture, adipogenesis was induced or not in MSC in presence of pesticides. Fourteen days later, the cultures were stained with Oil-Red and counterstained with hematoxylin. 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 percent adipocytes. ($n = 3$, $*p < .05$, $***p < .001$). Abbreviations: ADI, acceptable daily intake; hNDI, high nutritional daily intake.

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 Supporting Information 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 (Supporting Information 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 3.15–220 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 nutritional 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 tMSC 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 44 μM, with a recommended optimum level of about 50 μM [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.

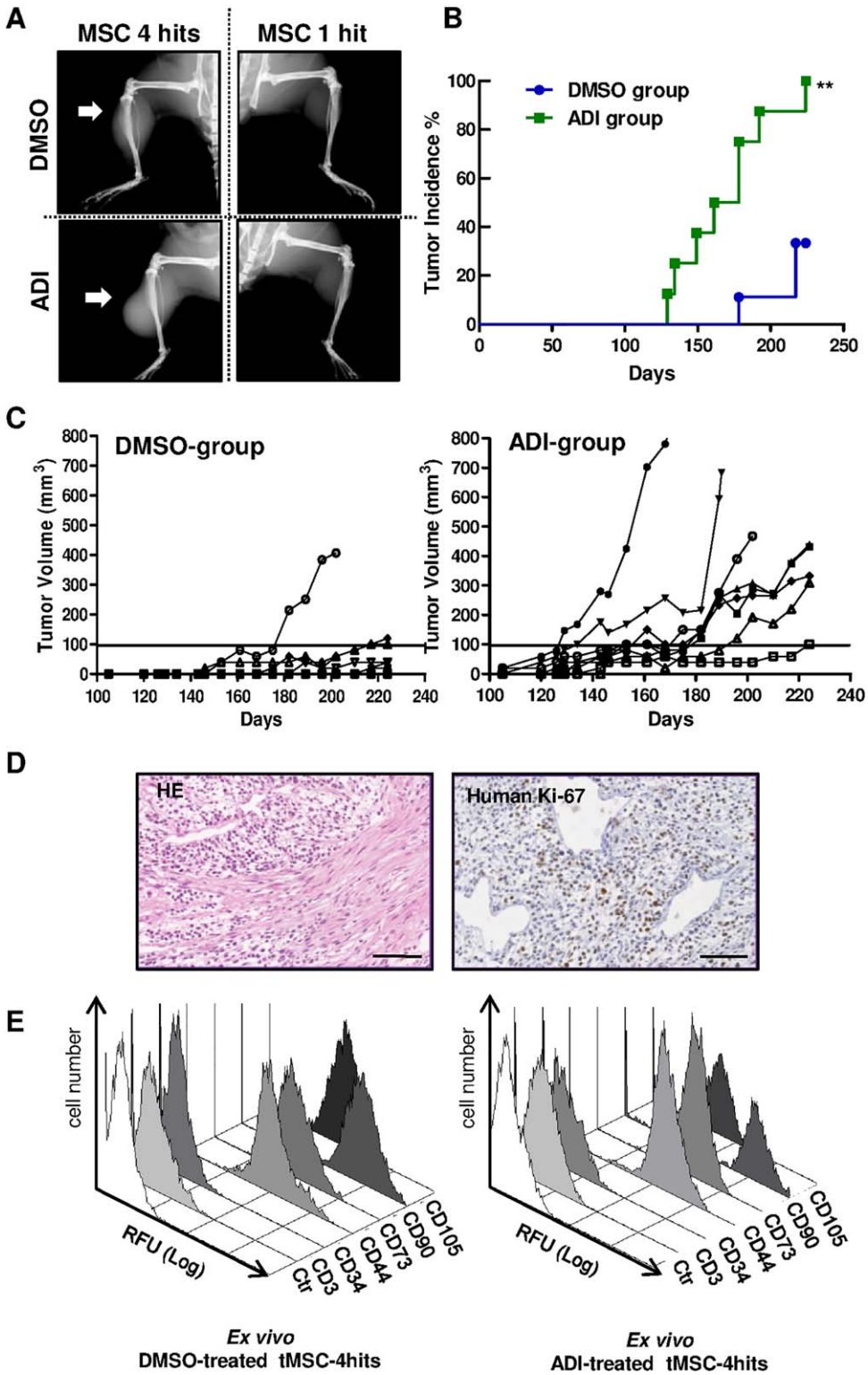

Figure 6. Pesticide mixture promotes the tumorigenic phenotype of tMSC in vitro and in vivo. **(A)**: Illustration of tumour growth in nude mice after paratibial implantation of tMSC-1hit or tMSC-4hits pre-treated for 21 days with DMSO (DMSO-group) or with pesticide mixture at ADI dose (ADI-group). **(B)**: Tumour incidence in both groups (three mice of nine in DMSO-group and eight mice of eight in ADI-group, $^{**}p = .011$). **(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 (100 μm). **(E)**: Flow cytometry analysis of ex vivo cultures of tumour cells from both DMSO- and ADI-groups showing positive staining to MSC stemness markers. Abbreviations: ADI, acceptable daily intake; HE, hematoxylin and eosin; MSC, mesenchymal stem cells; tMSC transformed mesenchymal stem cells.

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 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 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 three out of nine 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 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 pretreated with pesticide mixture induced undifferentiated high-grade pleomorphic sarcomas, similarly to tumours induced in mouse following *p53* and *Retinoblastoma protein (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 (Supporting Information Fig. 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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Author Contributions

C.O. and F.M.V.: developed the concepts and designed the experiments; M.H.: performed in vitro experiments with the help of L.O.; C.P. and M.H. performed the SeaHorse experiments and analyses; V.T., P.A., J.D., R.B., and J.A.: performed in vivo experiments and analyses; D.H. and P.N.: help to design in vivo experiments and to discuss results; M.H., C.O., and F.M.V. wrote the paper; all authors analyzed results, wrote the methods section, and edited the manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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