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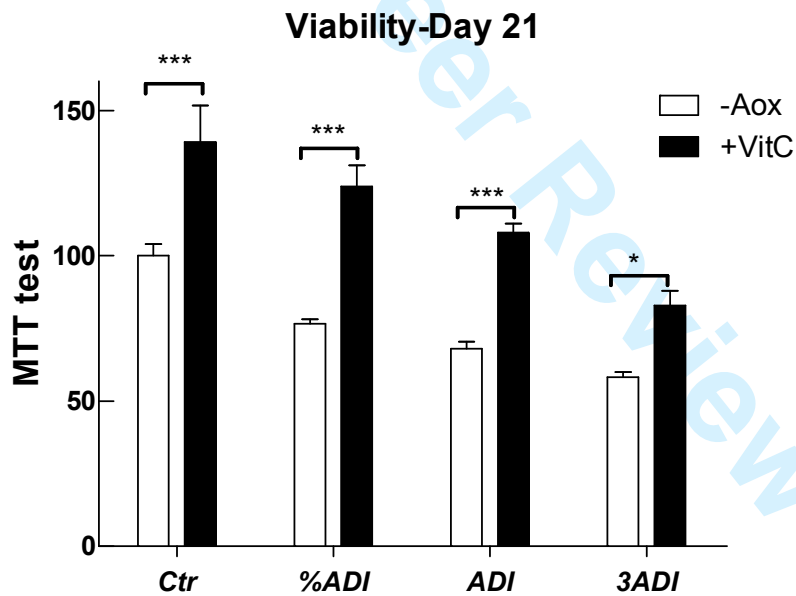
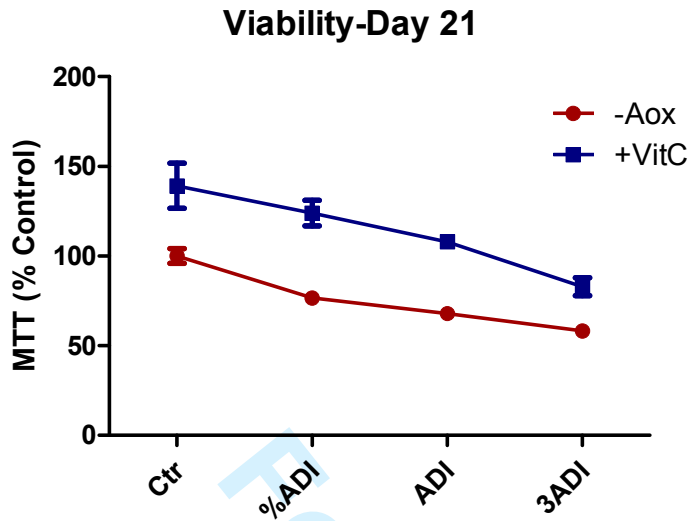
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Low-dose pesticide mixture induces senescence in normal mesenchymal stem cells (MSC) and promotes tumorigenic phenotype in premalignant MSC.

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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.

1 INTRODUCTION

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

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

1
2
3 27 exposure to high doses of mixtures of pesticides induced a direct hematotoxicity in the
4
5 28 hematopoietic niche and led to hypoplastic marrow[13]. Mesenchymal stem cells (MSC) are
6
7 29 important components of the hematopoietic niche and are necessary for niche homeostasis
8
9 30 and their absence reduces hematopoietic stem cell development and repopulation [14, 15].
10
11 31 MSC are also found in other compartments such as adipose tissue, skin, dental pulp and in the
12
13 32 circulation after bone marrow rupture [16]. They have the capacity to differentiate into many
14
15 33 cell types including osteoblasts and adipocytes [17]which enlarges their functional
16
17 34 contribution. Apart from their physiologic roles, MSC may promote tumour growth [18-20] or
18
19 35 metastasis [21].

20
21 36 The aim of the study was to investigate the impact of long-term exposure of MSC to mixture
22
23 37 of pesticides of different chemical families at low concentrations (nanomoles to a few
24
25 38 hundred nanomoles range) extrapolated from the residual quantities found in the western
26
27 39 European diet. For this purpose, we studied *in vitro*, the effect on MSC of a mixture of seven
28
29 40 pesticides (Chlorpyrifos-Ethyl, Dimethoate, Diazinon, Iprodione, Imazalil, Maneb and
30
31 41 Mancozeb) frequently detected in food samples[10]. We found that this combination induces
32
33 42 senescence *in vitro* and enhance tumorigenesis *in vivo*. Our results suggest that the
34
35 43 combination of small amounts of pesticides could accelerate age-related diseases in healthy
36
37 44 MSC and favour the onset of cancer in predisposed MSC.

45 MATERIAL AND METHODS

46 Materials

47 All pesticides (Chlorpyrifos Ethyl, Dimethoate, Diazinon, Iprodione, Imazalile, Maneb and
48
49 48 Mancozeb) as well as the senescence cell histochemical staining kit (SA-beta-Galactosidase)
50
51 49 were obtained from Sigma Aldrich (St. Louis, MO, USA). Foreskin-derived human
52
53 50 fibroblasts were obtained from the Department of Pediatrics at “Hôpital Mère et Enfant de
54
55 51 Nantes”. Human microvascular endothelial cells (HMVEC) were kindly provided by Dr
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3 52 François Paris (INSERM-U892, Nantes, France). Mesenchymal stem cells (MSC) were
4
5 53 obtained from human bone marrow aspirates which were kindly provided by Drs Philippe
6
7 54 Rosset and Louis-Romée Le Nail from Tours University Hospital (France) during orthopedic
8
9 55 surgical procedures. Oral consent was obtained from informed patients in accordance with
10
11 56 French law (Art. L. 1245-2 of the French public health code, Law n° 2004-800 of 6 August
12
13 57 2004, Official Journal of 7 August 2004). At least five batches of MSC obtained from
14
15 58 different young and healthy donors (aged of 23-35 years) were used. MSC were characterized
16
17 59 as previously described [22-24]. Briefly, the surface markers including CD34, CD45, CD73,
18
19 60 CD90, and CD105 and the differentiation capacity towards three lineages (osteoblast,
20
21 61 adipocyte, chondroblast or myoblast) were assessed and confirmed as recommended[25]. Cell
22
23 62 media: Alpha-MEM, DMEM, RPMI-1640, and fetal calf serum (#10270) were
24
25 63 from ThermoFisher Scientific (Villebon-sur-Yvette, France). Transformed mesenchymal stem
26
27 64 cells were kindly provided by the laboratory of Dr Tim Fenton (UCL Cancer Institute,
28
29 65 London, UK). MesenCult and the corresponding serum were from StemCell Technology
30
31 66 (Grenoble, France). Medium and supplements for HMVEC (EBM-2) were from Lonza
32
33 67 (Viviers, Belgium). The references of the antibodies used in this study are listed in Table S1.
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39 **Pesticides-mixture doses and preparation**

40
41 69 The doses of pesticides used were extrapolated, for each pesticide, from three values. First,
42
43 70 the high Nutritional Daily intake (hNDI), calculated for the French population of all-age,
44
45 71 based on the method of the EFSA [11]. hNDI are the very low doses of pesticides used in our
46
47 72 study (3 to 220 times less than the ADIs). The next doses are the international Acceptable
48
49 73 Daily Intakes (ADI), specified by the WHO or the European Commission, representing the
50
51 74 threshold of safety in humans for lifetime exposure. Last, a value of 3 times the ADI (3ADI)
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53 75 that we used as a positive control.
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3 76 For the hNDI, the method in which EFSA determined chronic exposure is comparable to the
4
5 77 calculation of the Theoretical Maximum daily Intake (TMDI) according to the following
6
7 78 equation:

9
10 79 $TMDI = \sum MRL_i \times F_i$

11
12 80 MRL_i : Maximum residue level for food commodity i ; F_i : Food consumption of food
13
14 81 commodity i

15
16 82 In order to introduce a more realistic estimation of the exposure to a pesticide, the EFSA
17
18 83 replaced the MRL_i values by the relevant residue concentration obtained on analyzed
19
20 84 samples. The calculated TMDI results were reported separately for each pesticide and for 27
21
22 85 different diets. The highest estimated exposure for each pesticide, expressed as percent of the
23
24 86 ADI, is reported for each diet. In France, three groups of diets were represented: Infant,
25
26 87 Toddler and general population according to the EFSA report 2010[10]. We calculated an
27
28 88 average value (in % ADI) that we converted to mg/Kg.bw/day considering the ADI value for
29
30 89 each pesticide. For the three values of hNDI, ADI and 3ADI, a further conversion has been
31
32 90 made in order to extrapolate the aforementioned doses (estimated in mg/Kg body weight) to
33
34 91 an in vitro model. In general, the amount of pesticide ingested daily is rapidly absorbed and
35
36 92 rapidly eliminated (in whole or in part) by the body. We considered the case of a total
37
38 93 absorption of this ingested amount and then its dilution in 5 liters of blood in a subject of 60
39
40 94 Kg, in order to obtain the blood concentration (mg/l) to which the various organs could be
41
42 95 theoretically exposed. Finally, from the molar mass of each pesticide, we calculated a
43
44 96 concentration in $\mu\text{mole/L}$.

45
46 97 Pesticides were dissolved in DMSO and mixtures were prepared at the three aforementioned
47
48 98 doses. Whatever the dose applied, the maximal volumes of DMSO \pm pesticides added to the
49
50 99 media did not exceed 1/1000 (v/v) of the medium. Throughout the study, cells were treated
51
52 100 with pesticides mixture for 21 days and media were changed every three days.
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101 Cells Culture and treatment

102 Except for the experiments on MSC differentiation, MSC were cultured at 2000 cells/cm² in
103 alpha-MEM modified with ribonucleosides and deoxyribonucleosides and supplemented with
104 fetal calf serum (FCS) (10%) and 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL
105 streptomycin and 0.2 ng/mL of FGF2 in an atmosphere of 5% CO₂ and 95% humidity at
106 37°C. MSC cultures were used between passages 4 and 7. Modified MSC (we called
107 Transformed MSC or tMSC) were cultured in complete MesenCult medium (StemCell
108 technology). Fibroblasts were cultured in RPMI-1640 medium and supplemented with FCS
109 (10 %) and 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (used
110 between passage 4 and 50). Human microvascular endothelial cells (HMVEC) cultures were
111 supplemented with standard commercial medium for endothelial cells (EBM-2) (Lonza).
112 Pesticide mixture at doses of hNDI, ADI or 3ADI were dissolved in DMSO and added to the
113 cell media, over the 21-day exposure. DMSO doses versus media did not exceed 1/1000 (v/v)
114 for control and pesticides-treated cells. In some experiments, the pefithrin- α (10 µM) (Sigma)
115 was used from day 7 until the end of the experiment. Media were replaced every 2-3 days.

116 Mitochondrial stress analysis

117 The mitochondrial stress and the glycolytic capacity of MSC were analyzed by the Seahorse
118 XF24 Flux Analyzer (Seahorse Bioscience, Copenhagen, Denmark). Briefly, at day 7 and day
119 21 after exposure to the pesticide mixture, cells were trypsinized and reseeded at 2x10⁴ cells
120 /well and left overnight without the pesticide mixture to adhere before the analysis. Cells were
121 equilibrated for 1 h at 37 °C in bicarbonate-free DMEM (Sigma) supplemented with 25 mM
122 glucose, 1 mM pyruvate and 2 mM Gln, pH was adjusted to 7.3 with NaOH before the
123 analysis. To determine mitochondrial parameters, Oxygen Consumption Rate was measured
124 at baseline and after respective addition of oligomycin (0.75 µM), CCCP (1.5 µM), rotenone
125 (1 µM) and antimycin A (1 µM). All measurements were done in five wells per condition.

1
2
3 126 Three to five independent experiments were conducted. Respiratory spare capacity was
4
5 127 calculated as maximal OCR, after oligomycin and CCCP injections, minus basal OCR.
6
7 128 Coupling efficiency corresponded to OCR inhibition by oligomycin. The basal glycolytic
8
9 129 capacity was extrapolated from the extracellular acidification rates values (ECAR, Δ pH/min)
10
11 130 of cell media before the addition of the aforementioned drugs.

131 **In vitro MSC differentiation**

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

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

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3 151 Ct method[26]. The reference genes RLP19 and GAPDH were used for normalization. For the
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5 152 adipogenic differentiation of MSC, cells were seeded in 24-well plates and on 4 chambers
6
7 153 glass slides (Labtek, Dominique Dutscher, Brumath, France) at 2×10^4 cells/cm² and
8
9 154 incubated with MSC Adipogenic Bullet kit (Lonza) for 21 days. The same scheme of
10
11 155 pesticides exposure was pursued as for osteogenic differentiation. Adipocytes were further
12
13 156 stained with Oil Red O staining (Sigma), and counterstained with hematoxylin. For 24-well
14
15 157 plates, images were acquired by Arrayscan VTI HCS reader (ThermoFisher Scientific) and for
16
17 158 the slides we used the whole-slide scanner NanoZoomer 2.0-HT (Hamamatsu).

159 **In vivo MSC injection**

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

175 **Statistical analysis**

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3 176 Results are expressed as mean \pm SEM. Statistical analysis was performed when appropriate
4
5 177 using one-way or two-way ANOVA followed by Tukey or Dunnett's multiple comparison
6
7 178 tests. For in vivo comparison "Gehan-Breslow-Wilcoxon Test" was used. Statistics were run
8
9 179 with GraphPad Prism®. Differences with $P < 0.05$ were considered statistically significant.

180 **Miscellaneous**

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

185 **RESULTS:**

186 **Specific induction of senescence in MSC by an environmental combination of** 187 **pesticides.**

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

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3 202 results support our contention of a theoretical additive response of our pesticide combination
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5 203 that is not the mere sum of individual effects and shows specific cellular response. MSC cell
6
7 204 death did not seem to be affected by the treatment at any combination concentration and no
8
9 205 apoptosis was observed at day 21 as no caspase-3 activity was detected (Fig.S4A). The
10
11 206 expression of some proteins of the BCL2-family was deregulated (i.e. BAX and MCL-1
12
13 207 expressions were decreased and BCL_{XL} was increased) which suggested an increased
14
15
16 208 resistance to apoptosis (Fig.1D and Fig.S2B). However, since the resistance to cell death in
17
18 209 undifferentiated MSC is usually high[27], the consequence of the treatments on the threshold
19
20 210 to apoptosis might not be efficient. Autophagy and senescence could protect the cell from the
21
22 211 toxicity of external stress such as pesticides [28, 29]. Basal autophagy is already important in
23
24 212 MSC [29], and no increase of the cleaved forms of LC3 and degradation of p62 independently
25
26 213 of the administered doses was observed (Fig.1E and Fig.S4C). Next, we determined if the
27
28 214 reduced cell number was due to senescence. An increase of senescent cells was established in
29
30 215 MSC in a dose-dependent manner (Fig.1F). MSC senescence was important even at very low
31
32 216 dose of pesticide mixture and ranged from 30% with hNDI to about 50% with 3ADI. Of note,
33
34 217 this effect was specific to MSC at these doses as we did not observe any induction of
35
36 218 senescence in fibroblasts or in endothelial cells after 3-week exposure to the pesticide mixture
37
38 219 (Fig.1G and H and Fig.S5, upper panel).

220 **Pesticide mixture does not induce a replicative senescence in MSC**

221 Cellular senescence may be replicative (telomere-dependant) or stress related. To further
222 study the pesticide-induced senescence, we used, in addition to naïve MSC, the modified
223 human adult MSC (tMSC) with genetic alterations described by Funes *et al.*,[30] (Fig.S6). In
224 naïve MSC, the pesticides induced an upregulation of p21^{waf1} but not in p53 expression at day
225 21 or earlier (Fig.2A and Fig S4D), while p16^{INK4a} was not detected by Western blot in these
226 cells under our conditions (Fig.S4B). In addition, the inhibition of the transcriptional activity

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2
3 227 of p53 by pifithrin- α (10 μ M from day 7) (Fig. S5, lower panel) or its invalidation in tMSC-
4
5 228 2hits (Fig.2B) completely abrogated the pesticides-induced senescence. In tMSC-1hit, the
6
7 229 ectopic expression of hTERT extends MSC lifespan in culture as previously described [30]. In
8
9 230 these cells, pesticides were able to induce dose-dependent senescence (from 15 % with hNDI
10
11 231 up to 40% with 3ADI) although with less efficiency than that observed in naïve MSC. Of
12
13 232 note, p21^{waf1} was upregulated in these cells (Fig.2C). Altogether, this suggests that pesticide-
14
15 233 induced senescence in MSC was p53 but not telomere dependent.
16
17

18 234 **Pesticide mixture induces a stress-related senescence in MSC.**

19
20 235 Oxidative stress is one of the classical initiators of senescence[31]. Pesticides can also induce
21
22 236 an oxidative stress in many cell types [32-35]. To analyse the effect of the pesticide
23
24 237 combination, the generation of reactive oxygen species (ROS) was analyzed at day 7, 14 (Fig.
25
26 238 S7A) and day 21 (Fig.3A). Our combination was able to generate a dose-dependent increase
27
28 239 of continuous ROS production in MSC even with the lowest concentrations used, persistent
29
30 240 until day 21(Fig.3A). ROS production in fibroblasts and endothelial cells was moderately
31
32 241 increased only at highest concentrations of pesticides in endothelial cells (3ADI, Fig.3A)
33
34 242 while no increase was detected in fibroblasts at day 21(Fig.3A).Of note, the basal levels of
35
36 243 ROS were nonetheless superior in endothelial cells and fibroblast compared to MSC. Since
37
38 244 the induction of ROS by our pesticide combination appeared to be specific to MSC, we
39
40 245 investigated further its mechanisms. The expression of various antioxidant enzymes was
41
42 246 evaluated by immunoblots in MSC and fibroblasts (Fig.3B) after 21-day exposure. In MSC,
43
44 247 hNDI dose induced an upregulation of the mitochondrial enzymes superoxide dismutase
45
46 248 SOD2 and glutathione peroxidase GPX1, whereas higher doses (ADI and 3ADI) led to a more
47
48 249 potent response with an additional increase in both cytosolic proteins GPX2 and catalase
49
50 250 expression. These effects were not related to a deterioration of the mitochondrial structure as
51
52 251 shown by the staining of functional mitochondria and the steady levels of citrate-synthase, a
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1
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3 252 marker of mitochondrial biogenesis (Fig.S7B). In fibroblasts, the enzymes were constitutively
4
5 253 expressed in control cells and no significant changes in the expression of SOD1/2, GPX1/2 or
6
7 254 catalase were seen (Fig. 3B). This antioxidant profile was consistent with the ROS production
8
9 255 profile. Moreover, vitamin C reduced ROS production in MSC treated or not with the
10
11 256 pesticide mixture at day-4 (80%) without affecting their viability (Fig.S7C). Treatment with
12
13 257 vitamin C, starting on day 7 during the 3-week exposure to pesticide mixture reversed the
14
15 258 pesticides-induced senescence in MSC and attenuated the expression of p21^{waf1} and p53 (Fig.
16
17 259 3C) without affecting cell viability (Fig.S7C). Altogether, these results indicate that our
18
19 260 pesticide mixture induced specifically a stress-related senescence in MSC that can be
20
21 261 overcome by vitamin C.

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

263 It has been reported that exposure to some pesticides, especially organophosphates, disturbed
264 the cell respiratory system[36]. Moreover, oxidative stress[37] as well as induction of
265 senescence[38] may alter the cellular metabolic capacities. So,we investigated the impact of
266 our pesticide mixture on the mitochondrial respiratory profile of MSC and their basal
267 glycolysis early on day 7 and on day 21. While a global reduction in both mitochondrial
268 capacity and glycolysis was observed at day 7 (Fig.S8), the global metabolic profile was
269 completely different on day 21. Basal mitochondrial respiration was restored at doses of hNDI
270 and ADI and even increased in 3ADI-treated cells. A similar effect was observed with
271 OXPHOS-coupled ATP production (Fig.4A). The expression of ATP synthase was also
272 increased (Fig. 4B). However, pesticides-treated MSC showed an important use of their spare
273 capacity, which is the extra capacity available in cells to produce energy in response to
274 increased stress (spare capacities were 38.10±5.6 in Control MSC versus 15.69±4.05,
275 19.93±5.14, 10.77±2.78 with hNDI, ADI and 3ADI respectively, $p<0.05$). Proton leak was
276 unchanged. The basal glycolysis in MSC, a main energy source for MSC *in vivo*[39] was not

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2
3 277 significantly different from control cells (Fig.4C). Altogether, the late respiratory features
4
5 278 show an adaptation of MSC, despite the continuous presence of pesticides at this dose range,
6
7 279 leading to an elevation of OXPHOS at 3ADI. Moreover, mitochondria functioned at full
8
9 280 capability to ensure sufficient metabolic resources losing their spare capacity, which is
10
11 281 necessary to face environmental stress and increased energetic demands.
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13

14 282 **Pesticides alters the differentiation in MSC**

15
16 283 One important specificity of MSC is their capacity to differentiate into other cell types like
17
18 284 osteocytes and adipocytes. However, little is known about the effect of pesticides, either in
19
20 285 mixtures or alone, on the differentiation of MSC. A 21-day treatment with pesticide mixture
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22 286 did not alter the MSC stemness as they continued to express OCT-4 and SOX-2 (Fig S9A)
23
24 287 with an increase in SOX-2 expression. We induced osteogenic or adipogenic differentiation in
25
26 288 MSC in the presence of pesticides during 21 days. MSC showed lower tendency to osteogenic
27
28 289 differentiation at low doses (hNDI and ADI), and this was significant with 3ADI as seen with
29
30 290 Red-Alizarin staining (Fig.S9B). This was accompanied by a decreased expression of *RUNX2*
31
32 291 transcript, a major transcription factor associated with the induction and commitment to
33
34 292 osteogenesis, on day 7. In contrast, the expression of *ALP* transcripts was not significantly
35
36 293 changed (Fig.S9C). On the other hand, the adipogenic differentiation was assessed and
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38 294 showed a significant dose-dependent increase in presence of pesticide mixture even with the
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40 295 lowest concentrations (Fig.5).
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45 296 **Pesticide mixture promotes tumorigenic phenotype in modified MSC**

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47 297 Pesticide exposure was associated with high risk of cancers [3, 40, 41], in addition, cellular
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49 298 senescence may constitute a permissive environment to cancer development[42]. To examine
50
51 299 the capacity of the low-dose pesticide mixture to induce malignant transformation in MSC,
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53 300 we tested the anchorage-independent growth of MSC treated with the pesticide mixture for 21
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55 301 days. Naïve MSC were unable to form colonies in soft agar whether they were pre-treated or
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3 302 not with pesticide mixture (Fig.S10A and table.S3) which indicates that pesticide mixture
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5 303 alone could not initiate tumorigenesis in normal MSC. We looked further to see if the
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7 304 pesticide mixture could promote a tumoral phenotype in modified tMSC. For this purpose,
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9 305 tMSC which were transduced by 1 to 4 oncogenic hits were used. A 21-day pre-treatment
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11 306 with pesticide mixture enhanced the colony formation after 28 days in the soft agar assay
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13 307 (Fig.S10A and table.S3). However, this effect was not dose dependent but was already
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15 308 important at the very low dose of pesticides (hNDI).
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17 309 In order to evaluate this effect *in vivo*, we treated both tMSC-1hit and tMSC-4hits either with
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19 310 DMSO or with our pesticide mixture at ADI dose. The cells were separately implanted
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21 311 bilaterally in *nude* mice paratibial zones. The first group was implanted with cells treated with
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23 312 DMSO (DMSO-group) and the second group with pesticide mixture-treated cells (ADI-
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25 313 group). Strikingly, only 3 of 9 mice showed tumour growth in DMSO-group all over the
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27 314 experiment while all the mice in ADI-group developed tumours ($p = 0.011$). In both cases,
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29 315 tumours were only observed with tMSC-4hits (Fig.6A and 6B), which corroborates the
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31 316 observation on tMSC clonogenicity *in vitro*. Furthermore, tumorigenesis showed accelerated
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33 317 kinetics of apparition in ADI-group compared to DMSO-group with onset after $160.14 \pm$
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35 318 31.62 days versus 204 ± 22.52 days respectively (Fig.6C). Histological analysis of tumours
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37 319 obtained from the ADI-group, showed undifferentiated connective tissues with fibroblastic
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39 320 and mixoid zones (HE staining, Fig.6D). Within these pleomorphic sarcomas induced by
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41 321 ADI-treated MSC-4hits, specific staining against human Ki-67 revealed a high proportion
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43 322 ($>13 \% \pm 6$) of proliferating human cells (Fig.6D). *Ex vivo* culture of tumour cells were
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45 323 prepared and analyzed by flow cytometry for cluster differentiation markers (CD) that are
46
47 324 characteristic of MSC. Cells derived from the tumours of ADI-group showed a CD expression
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49 325 pattern similar to MSC (negative for CD34 and CD45 and positive for CD44, CD73, CD90,
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51 326 CD105), the same pattern found in tMSC-4hits before any treatment (Fig.6E, Fig.S10C).
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3 327 Altogether, this indicates that the 21-day exposure to pesticide mixture was sufficient to
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5 328 induce long-lasting phenotypic changes in tMSC-4hits exacerbating and accelerating their
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7 329 tumorigenic transformation.
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9 330 **DISCUSSION**

10 331 The population is exposed in a chronic manner to multiple residues of pesticides belonging to
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12 332 diverse chemical families by, among others, the nutritional contamination. The EFSA report
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14 333 has shown that about 27% fruits, vegetables and commodities in Europe were contaminated
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16 334 with heterogeneous residues of pesticides simultaneously[10, 11]. The seven pesticides we
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18 335 chose (depicted in table S2) were among those frequently used and detected on food and
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20 336 commodities, according to the EFSA's report of 2010 and still feature in the recent report of
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22 337 2013 (See supplemental Fig.S1). In addition, they may be simultaneously present on different
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24 338 fruits and vegetables (like apple, tomato, cabbage, lettuce and pepper) and some of them like
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26 339 mancozeb, diazinon, Chlorpyrifos and the dithiocarbamates were associated with hematologic
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28 340 disturbances in humans[43, 44] and in animal studies.[13, 45, 46] We used, for our *in vitro*
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30 341 model, an experimental estimation of the nutrition-conveyed concentrations of pesticides to
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32 342 which the population may be exposed considering the average of the highest calculated
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34 343 exposures of all-age French population according to the EFSA's method. The final *in vitro*
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36 344 concentrations correspond to those obtained through one-compartment pharmacokinetic
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38 345 model for hNDI, ADI and 3ADI doses. The selected hNDIs, with doses 3.15 to 220 times
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40 346 inferior than their chronic risk thresholds, i.e. ADIs, only correspond to the maximal risk
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42 347 related to food ingestion, while the major population is exposed to divers other sources
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44 348 (through household use, environmental contamination, drinking water...etc). In addition,
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46 349 active principles were used in this study while pesticides are usually combined with adjuvants
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48 350 that may increase their toxic effects[47].
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3 351 One of our major observations is a dose-dependent induction of senescence in MSC after 21-
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5 352 day exposure to pesticide mixture at notional doses. This effect seemed selective to MSC but
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7 353 not to other cells such as endothelial cells and fibroblasts. On one hand, induction of
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9 354 senescence was important in MSC even with the lowest doses (about 30% cells) and was
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11 355 consistent with the dose-dependent decrease in number of proliferating cells at day 21, taking
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13 356 into consideration that the pesticide mixture did not alter the viability or induce death in MSC.
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15
16 357 On the other hand, pesticides were able to induce senescence and p21^{WAF1} upregulation in
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18 358 transformed MSC in which the human telomerase (hTERT) was constitutively expressed, but
19
20 359 the percentages of senescent tMSC were less than those in naïve MSC. Although this may be
21
22 360 due to the transduction of these cells, it may also suggest that only few MSC may have
23
24 361 undergone a telomere-dependent aging. Altogether, our results indicate that pesticides-
25
26 362 induced senescence was p53-dependent and mainly stress-related.
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29 363 Pesticides are known to cause oxidative stress in different cell types[34] and this may be a
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31 364 possible starting point for senescence[31]. The mixture led to a continuous dose-dependent
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33 365 ROS production in MSC whereas fibroblasts and endothelial cells appeared less sensitive.
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35 366 Interestingly, pesticides-treatment showed a rather dose-dependent activation of antioxidant
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37 367 enzymes; while hNDI upregulated mitochondrial SOD2 and GPX1, ADI and 3ADI doses
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39 368 were more aggressive and induced cytosolic enzymes too (GPX2, SOD1 and catalase).
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41 369 Nonetheless, it is important to note that only a high concentration of vitamin C (200 µM) was
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43 370 sufficient to reverse pesticide mixture-induced oxidative stress in MSC, while the plasma
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45 371 concentrations of vitamin C in the general population seem to vary, in function of age and
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47 372 health, between 31 and 44µM, with a recommended optimum level of about 50µM[48],
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49 373 suggesting that physiologic concentrations may not be sufficient to counteract the pesticides-
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51 374 induced senescence generated in MSC.
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3 375 Pesticides exposure has been reported to induce metabolic alterations [36, 49]. After 21-day
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5 376 exposure to pesticide mixture, MSC adapted to the prolonged aggression as they restored
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7 377 basal glycolysis and showed even more important OCR and mitochondrial ATP production
8
9 378 with an upregulation of ATP synthase. However, the decrease in mitochondrial spare capacity
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11 379 persisted. As this capacity can be used by the cell in order to face an environmental stress or
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13 380 increased energetic demands[50], the persistent reduction in MSC's spare capacity, with
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15 381 continuous exposure to pesticide mixture, suggests that MSC may become more vulnerable if
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17 382 further aggressions occur.

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20 383 Exposure of MSC during 21 days to pesticide mixture did not seem to alter their stemness as
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22 384 MSC continued to express both OCT4 and SOX2 with even higher levels of SOX2 as seen by
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24 385 immunoblot analysis. Neither the impact of pesticide mixture on SOX2 expression nor a
25
26 386 potential link between stemness markers and senescence in MSC are clear. However, in
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28 387 neural stem cells, SOX2 overexpression induced senescence [51].

29
30 388 On the other side, when MSC were committed to differentiation media, their differentiation
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32 389 potential of MSC into adipogenic or osteogenic lineage was disturbed by pesticide mixture. In
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34 390 fact, MSC showed lower tendency to differentiate into osteocytes while their adipogenic
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36 391 potential was raised. It is noteworthy that during aging, bone marrow is replaced by adipose
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38 392 tissue and shows progressive bone fragility [52]. Adipose replacement also plays an important
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40 393 role in haematopoietic alteration during aging [53].

41
42 394 Many epidemiologic reports associate pesticide exposure and cancers [3, 40, 41]. Exposure to
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44 395 our pesticide mixture was unable to initiate tumorigenesis in normal MSC *in vitro*. However,
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46 396 in modified tMSC developed by Funes JM *et al*[30], in which a stepwise oncogenic
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48 397 transformation was induced, the exposure to pesticide mixture increased the colony formation
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50 398 in tMSC-3hits and tMSC-4hits but not in tMSC-1hit expressing human telomerase (hTERT)
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52 399 only, as shown by soft agar assay. *In vivo* implantation in paratibial zone in nude mice
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3 400 showed that 21-day pre-treatment with pesticide mixture at ADI dose shifted tMSC-4hits into
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5 401 more tumorigenic phenotype as tumours appeared in all mice implanted with ADI-treated
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7 402 tMSC-4hits compared to 3/9 mice injected with DMSO-treated tMSC-4hits. The onset of
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9 403 tumour growth was also earlier in ADI-group (169 days median) compared to DMSO-group
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11 404 (217 days median). It is noteworthy that in the study of Funes JM *et al*, tMSC-4hits did not
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13 405 show tumour growth when injected subcutaneously in nude mice after 6-month follow-up and
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15 406 a fifth hit was necessary to achieve their complete oncogenic transformation[30]. The tumour
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17 407 growth in DMSO-group found in our study may be due to a different site of implantation. It is
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19 408 important to point out that tMSC-1hit did not develop any tumours. The last observation is in
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21 409 line with the *in vitro* colony formation.
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26 410 Human MSC-4hits pre-treated with pesticide mixture induced undifferentiated high-grade
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28 411 pleomorphic sarcomas, similarly to tumours induced in mouse following *p53* and *RB*
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30 412 deficiency[54]. Ex vivo analysis of tumour cells showed that tMSC-4hits treated or not with
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32 413 pesticide mixture remained undifferentiated and similar to parental cells (supplementary
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34 414 Figure S10B). MSC have been proposed as the cell of origin of several human soft tissue
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36 415 sarcomas including leiomyosarcoma and osteosarcoma but this was mostly proved in animal
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38 416 studies based on mouse MSC derived from *p53* and/or *RB* deficient models[55, 56]. Our data
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40 417 using human premalignant MSC indicate that pesticide-stress may enhance tumorigenesis in
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42 418 already transformed cells by inducing additional step towards a complete malignancy.
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46 419 In conclusion, we show that a mixture of pesticides that can be ingested on a daily basis may
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48 420 constitute an additional environmental factor favouring aging and aging-associated
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50 421 pathogenesis in normal adult stem cells and promote tumorigenesis in a predisposed stromal
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52 422 environment.
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54 423 **ACKNOWLEDGMENTS**

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22
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26 435 **CONFLICT OF INTEREST**

27 436 The authors declare no conflict of interest.

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563 LEGENDS TO FIGURES

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3 564 **Fig.1, Effect of pesticide mixture on fibroblasts, endothelial cells and MSC. (A)**
4
5 565 Endothelial cells were treated or not with pesticide mixture for 21 days and cell growth was
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7 566 analyzed by MTT at day 21 (n=3). **(B)** Fibroblasts were exposed to pesticide mixture for 21
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9 567 days and assayed as endothelial cells (n=3). **(C)** MSC proliferation in the absence or in the
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11 568 presence of pesticide mixture for 21 days. MSC were seeded at 2000 cells/cm² and treated
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13 569 with DMSO 1/1000 v/v (Ctr) or pesticides mixture for 21 days. Cell viability was analyzed by
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15 570 Propidium iodide (PI) staining ,and proliferation by BrdU incorporation the end of the
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17 571 experiment, n=3, * $p < 0.05$, ** $p < 0.01$ vs Ctr. **(D)** The expression of pro and anti-apoptotic
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19 572 proteins in MSC was analyzed by immunoblot after 21-day exposure to pesticide mixture
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21 573 (n=3). **(E)** LC3 β cleavage and p62/SQSTM1 degradation were detected by immunoblot
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23 574 analysis at day 21 after pesticide mixture treatment (n=3). **(F)** β -gal staining of MSC after 21-
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25 575 day exposure to pesticide mixture. The percentages of senescent cells were then determined.
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27 576 (n=3, $p < 0.01$ vs Ctr). **(G)** Endothelial cells and **(H)** fibroblasts were treated with pesticide
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29 577 mixture for 21 days then β -gal activity was determined (n=3) by β -gal staining (n=3).
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36 579 **Fig.2 Pesticide mixtures induce p53-dependant but not telomere-dependant senescence**
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38 580 **in MSC. (A)** Immunoblotting and semi-quantification of p21^{waf1} and p53 in MSC at day 21
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40 581 after exposure to pesticide mixture (n=3). **(B)** β -gal staining and quantification in transformed
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42 582 tMSC-2hits (hTERT⁺/p53⁻) 21 days after pesticide exposure (n=3). **(C)** β -gal staining in
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44 583 tMSC-1hit (hTERT⁺) in presence of pesticides at day 21. The percents of senescent cells in
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46 584 tMSC hTERT⁺ cultures were assessed for each condition and the immunoblot of the
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48 585 expression of p21^{WAF1} at day 21 was analyzed (n=3, * $p < 0.05$, ** $p < 0.01$).
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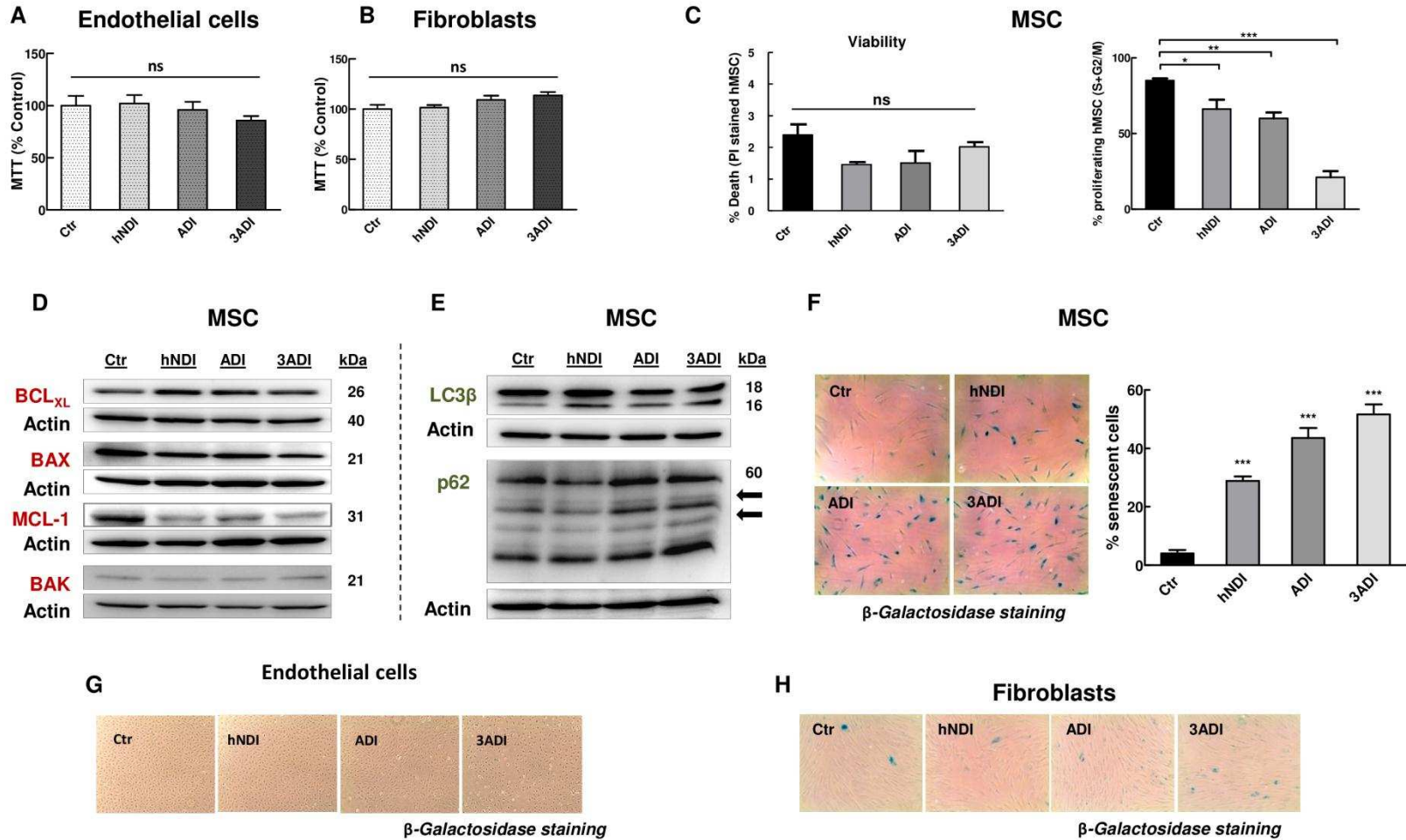
52 586 **Fig.3 Pesticide mixture enhances ROS production specifically in MSC and oxidative**
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54 587 **stress reversion abrogates pesticide-induced senescence in MSC. (A)** ROS production was
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56 588 analyzed in MSC, fibroblasts and endothelial cells using CMH₂-DCFDA at day 21 (n= 3,
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3 589 * $p < 0.05$, ** $p < 0.01$). (B) Expression of mitochondrial (SOD2 and GPX1) and cytosolic
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5 590 (SOD1, GPX2 and catalase) after exposure to pesticide mixture for 21 days by immunoblot in
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7 591 MSC (left panel) and fibroblasts (right panel) (n=3). (C) β -gal staining and quantification of
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9 592 MSC after 21-day exposure to pesticide mixture in the presence of vitamin C (200 μ M) from
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11 593 day 7 (n=3) (upper images). ROS activity in MSC in presence of vitamin C at day 21
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13 594 (** $p < 0.01$ versus vitamin C treatment, # $p < 0.05$ Ctr versus pesticide-treatment, n=3),
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15 595 expression of p21^{WAF1} and p53 under the same conditions (n=3).
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21 597 **Fig.4 Pesticide mixture causes respiratory changes in MSC.** (A) Mitochondrial respiration
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23 598 was assessed using the Seahorse XF24 Flux Analyzer. After 21 days of exposure to pesticide
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25 599 mixture, cells (2×10^4) were seeded in 24-well XF plate and the oxygen consumption rate
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27 600 (OCR) was measured before drug addition (basal respiration), mitochondrial ATP production
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29 601 was estimated after oligomycin treatment, proton leak was the result of the excess OCR
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31 602 between basal OCR and values after oligomycin addition. The % spare respiratory capacity
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33 603 was calculated after CCCP treatment. (B) The expression of ATP synthase (subunit- α) was
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35 604 analyzed by immunoblot at day 21. (C) Basal glycolysis was estimated from the ExtraCellular
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37 605 Acidification Rate (ECAR) before addition of drugs. (n=3, * $p < 0.05$, ** $p < 0.01$).
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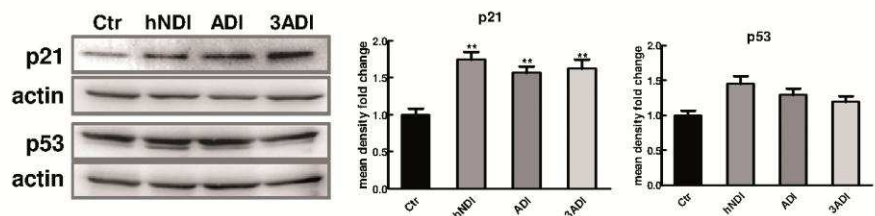
43 607 **Fig.5 Pesticide mixture promotes adipogenic transformation in MSC.** After 1-week
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45 608 treatment with pesticide mixture, adipogenesis was induced or not in MSC in presence of
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47 609 pesticides. Fourteen days later, the cultures were stained with Oil-Red and counterstained
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49 610 with hematoxylin. The photos are representative pictographs of the different cultures (bar of
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51 611 200 μ m). The percent of adipocytes was calculated either manually or after scanning with
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53 612 HCS Studio software. Results are presented as % adipocytes. (n=3, * $p < 0.05$, *** $p < 0.001$)
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3 614 **Fig.6 Pesticide mixture promotes the tumorigenic phenotype of tMSC *in vitro* and *in vivo*.**
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5 615 (A) Illustration of tumour growth in nude mice after paratibial implantation of tMSC-1hit or
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7 616 tMSC-4hits pre-treated for 21 days with DMSO (DMSO-group) or with pesticide mixture at
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9 617 ADI dose (ADI-group). (B) Tumour incidence in both groups (3 mice of 9 in DMSO-group
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11 618 and 8 mice of 8 in ADI-group, $**p=0.011$). (C) Kinetics of tumour apparition and growth in
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13 619 DMSO-group and ADI-group indicating the onset and the tumour growth in each mouse,
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15 620 mice were included when tumour volume attained 100 mm^3 . (D) Histological analyses of
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17 621 tumours obtained from ADI-group showing soft tissue sarcoma (HE staining) and
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19 622 proliferating human cells specifically detected with anti-human Ki-67 ($100\mu\text{m}$). (E) Flow
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21 623 cytometry analysis of *ex vivo* cultures of tumour cells from both DMSO- and ADI-groups
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23 624 showing positive staining to MSC stemness markers.
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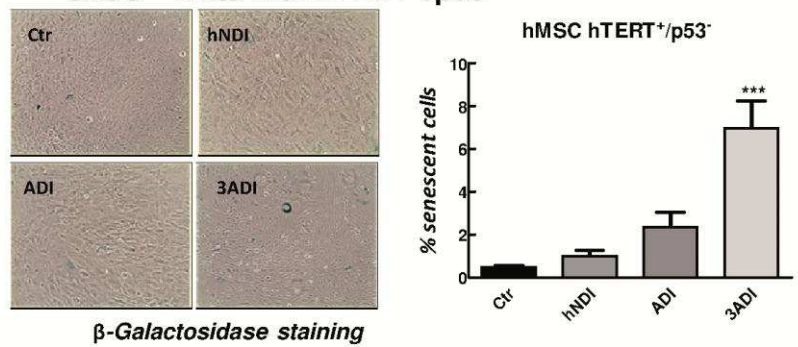


Stem Cells

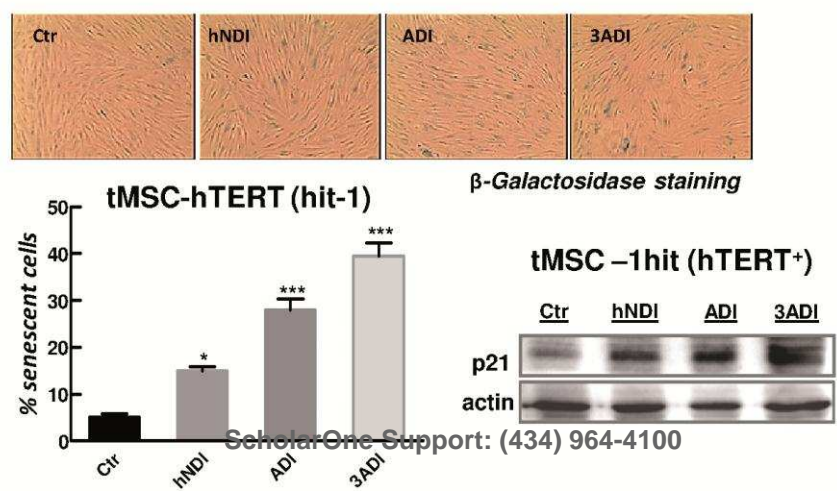
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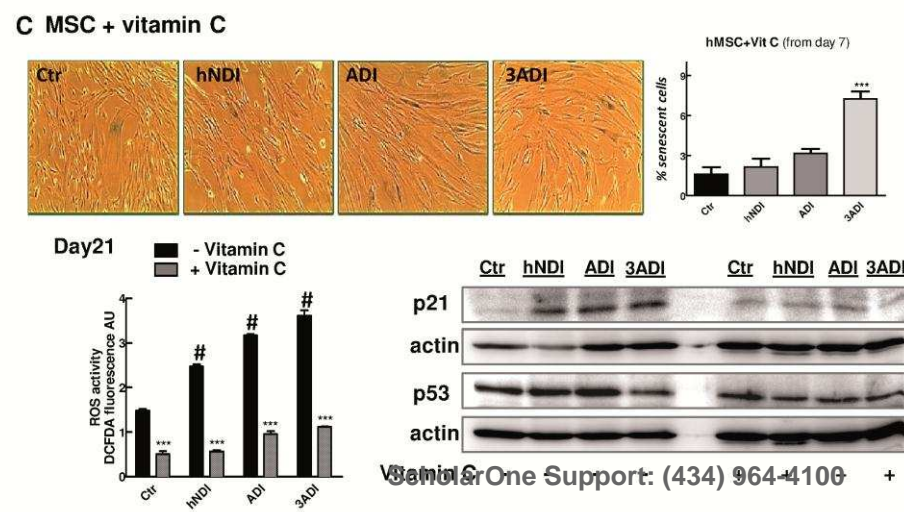
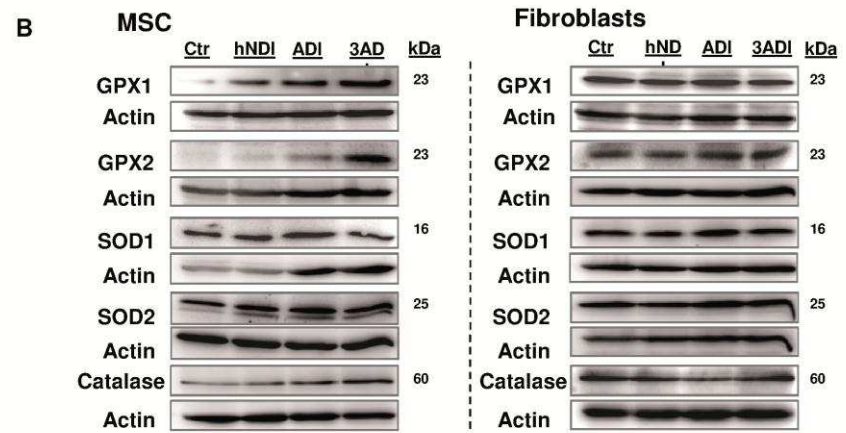
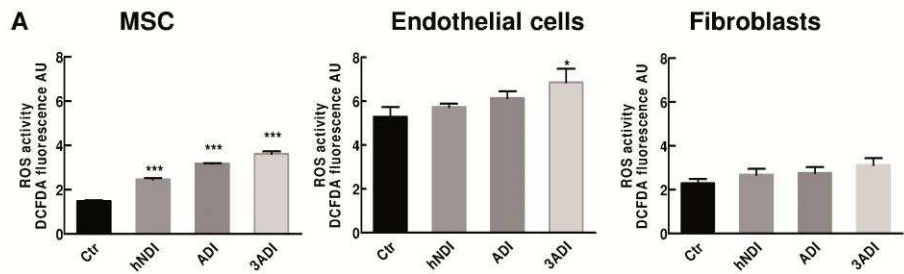
B tMSC – 2hits with hTERT+ /p53-



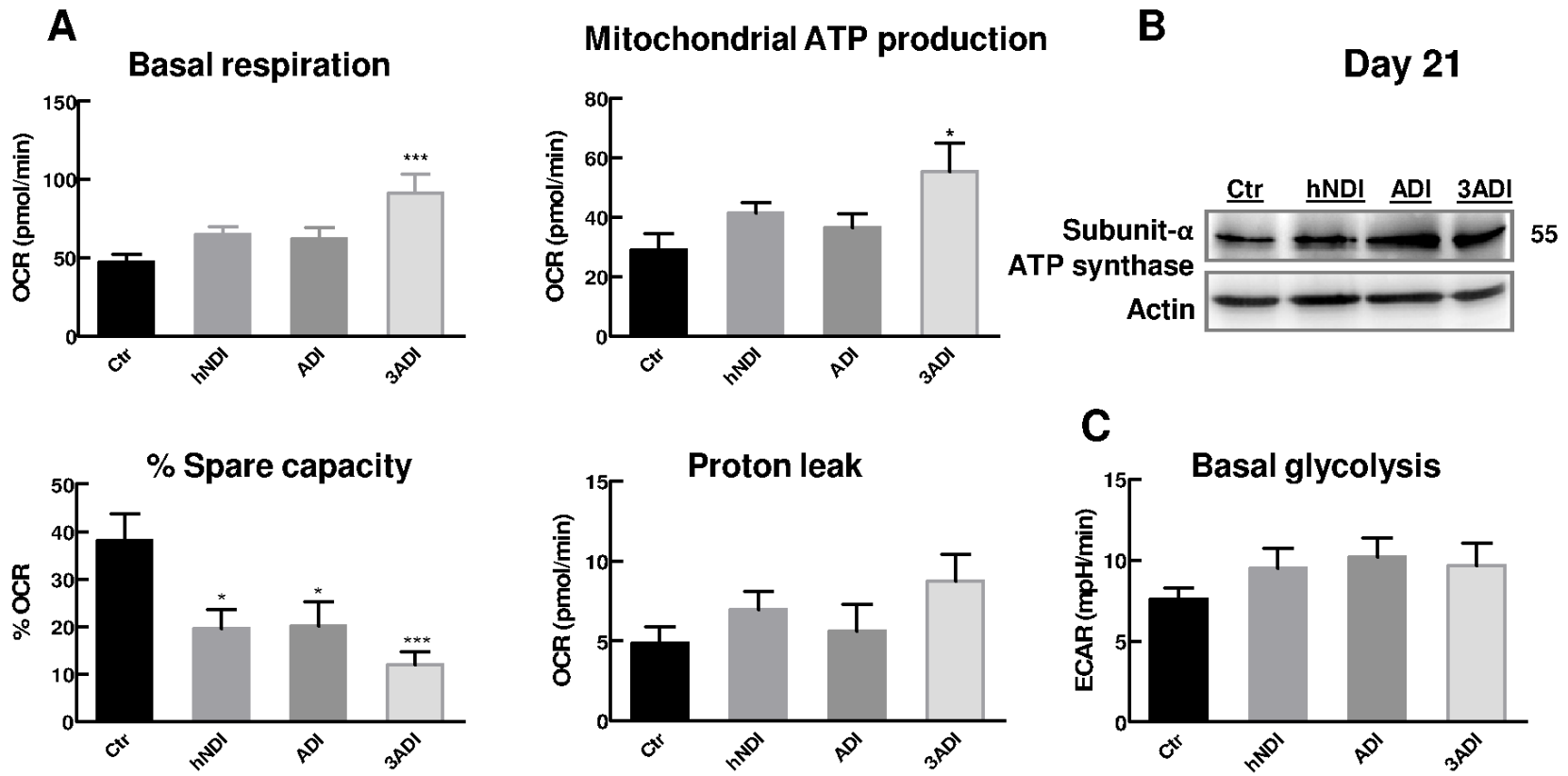
C tMSC –1hit (hTERT+)



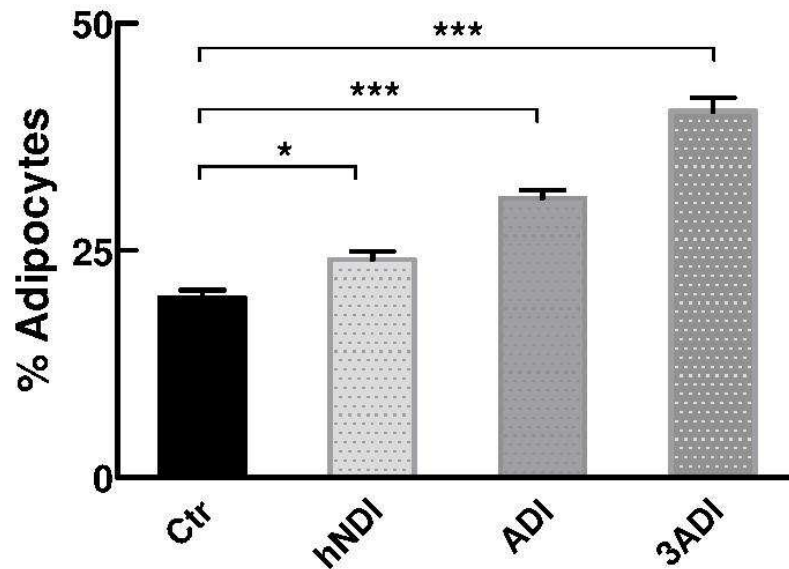
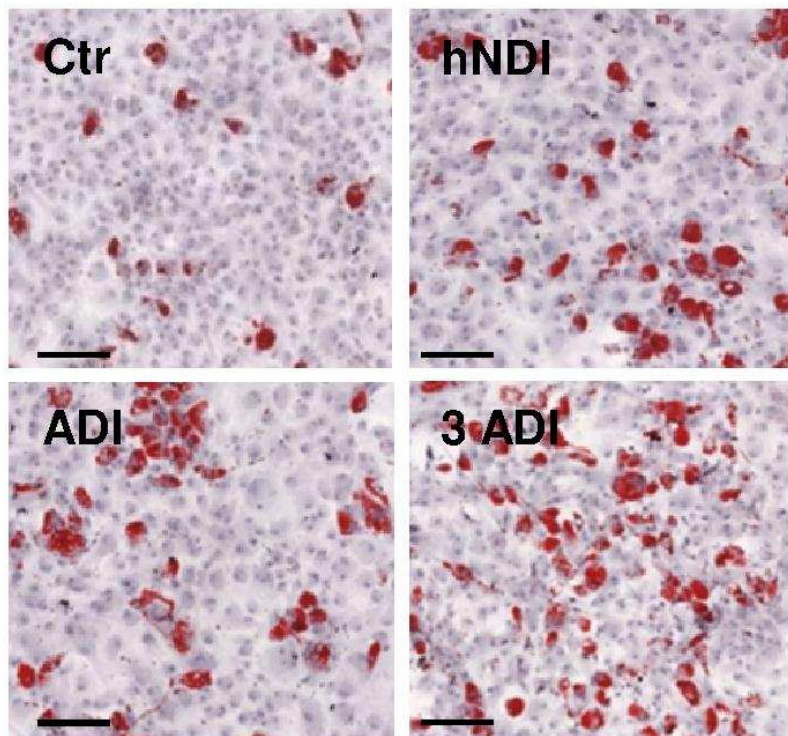
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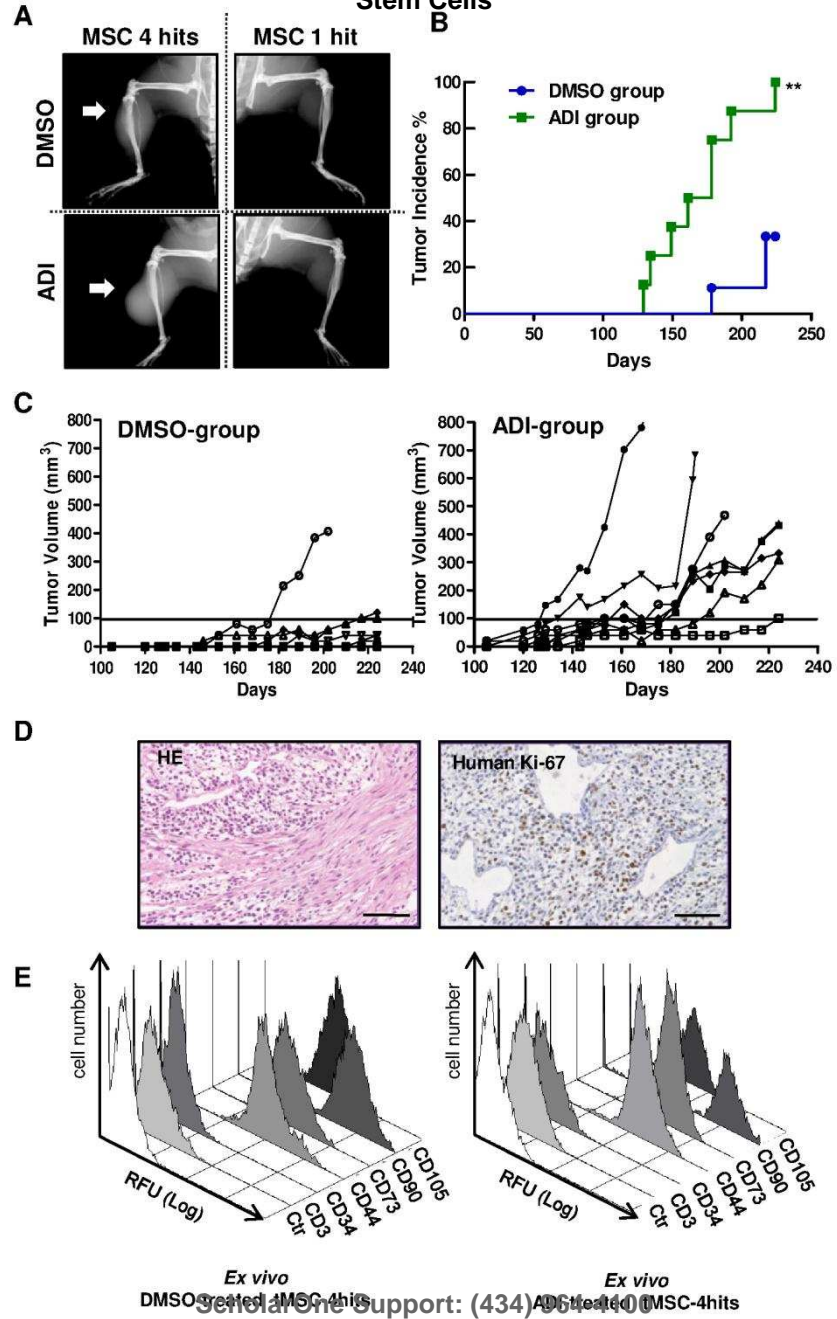


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Stem Cells



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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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Supplementary data:

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Supplementary methods

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Figures S1-S10

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Tables S1-S3

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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 μ l 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 μ l DMSO (Sigma). Aliquots of 100 μ l 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 μ g/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 μ g/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 (200 μ g/mL)/PI (10 μ g/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.

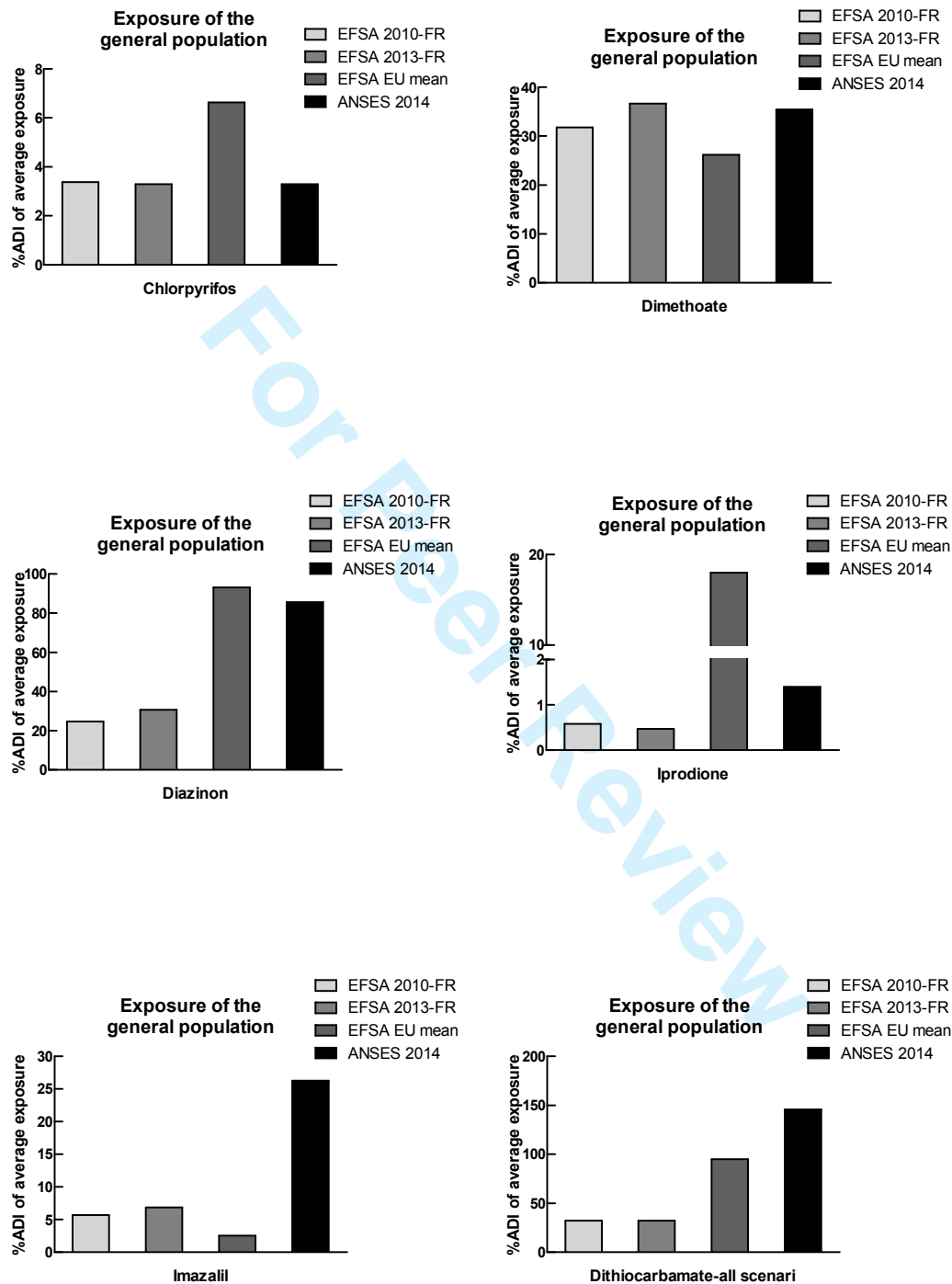
Histology analysis

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

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3 95°C within citrate buffer 1X pH 6.0 (MicromMicrotech) for 20 min, rinsed in PBS and
4 treated with 3% H₂O₂ before primary antibody. Overnight incubation at 4 °C with anti-
5 human Ki-67 (see antibody table) was performed with Tris Buffered Saline (TBS 1X pH
6 7.6, tween 0.05 %, triton 0.1%, bovine serum albumin 1% and 2% of goat serum). A
7 biotinylated goat anti-mouse IgG secondary antibody (1/400, E0433, Dako) was added
8 and detected with Streptavidin/HRP (horse radish peroxydase, Dako) complexes which
9 were revealed by a short incubation with 3,3'- Diaminobenzidine (DAB, Dako). Nuclei
10 were counterstained with a Gill-Haematoxylin solution. The proportion of Ki67-positive
11 cells was calculated using ImageJ software (NIH, Bethesda, MD, USA) from counting
12 >15000 nuclei in 3 tumor sections.
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17 **Flow cytometry analysis**

18 For phenotypic characterization, ex vivo cultures were prepared from tumors as following:
19 tumors were dissected and tumor fragments were then dilacerated in a FCS-deprived
20 medium before being incubated with Collagenase A (1 mg/mL) for 90 min under circular
21 agitation. The fragments were then filtered (70 µ) and mashed and washed with MSC
22 complete medium, centrifuged at 500 g for 10 min. the supernatants, rich with cells, were
23 then put in culture. About 105 cells (MSC or tumor-derived cells) were suspended in 50
24 µL phosphate buffered saline (PBS), 0.5% BSA , 2 mM EDTA and 1 µL of one of the
25 following antibodies: PE-conjugated mouse anti-human CD34 (clone 563), CD44 (clone
26 515), CD45 (clone HI30) or CD73 (clone AD2) from BD Biosciences (Le Pont de Claix,
27 France) or PE-conjugated mouse anti-human CD90 (clone5E10) or CD105 (clone 43A3)
28 from BioLegend through Ozyme (Paris, France). After washing in PBS, cells were
29 analyzed with flow cytometer (Beckman Coulter FC500).
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FIGURE S1**Fig. S1.**

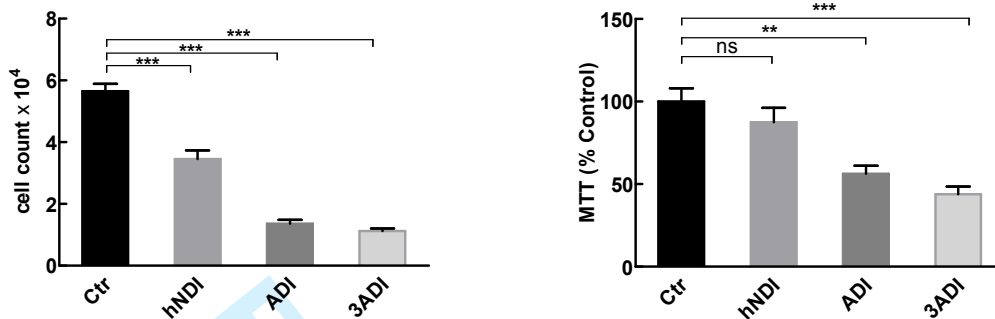
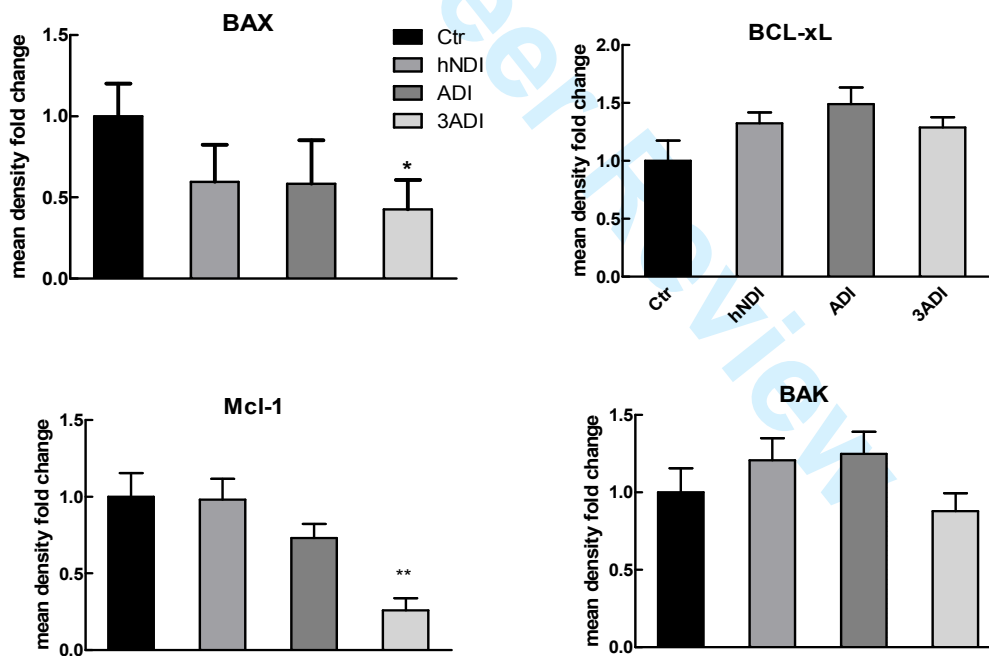
Mean quantities of pesticides residues found in French and European food as reported by EFSA 2010, EFSA 2013 and ANSES 2014. Values are presented for each pesticide as %

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3 of its ADI. The mean quantities of our 7 pesticides (Chlorpyrifos, Diazinon, Dimethoate,
4 Iprodione, Imazalil, Maneb and Mancozeb) reported by three major studies (EFSA,2010¹
5 and 2013² for French and European general populations) and ANSES, 2014³
6 (*Agence Nationale de Sécurité Sanitaire de l'Alimentation*, for French population). In
7 these reports, the values of the mean chronic exposures were calculated for each pesticide
8 as percents of its Acceptable Daily Intake (ADI). For maneb and mancozeb, results were
9 depicted as Dithiocarbamate-all scenari because the method of detection makes no
10 specific distinction between these two compounds. In our study, we used the values
11 reported by EFSA,2010.

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14 1- http://www.eurosfairerpr.fr/7pc/documents/1279027807_rapport_efsa_15_juin_2010.pdf

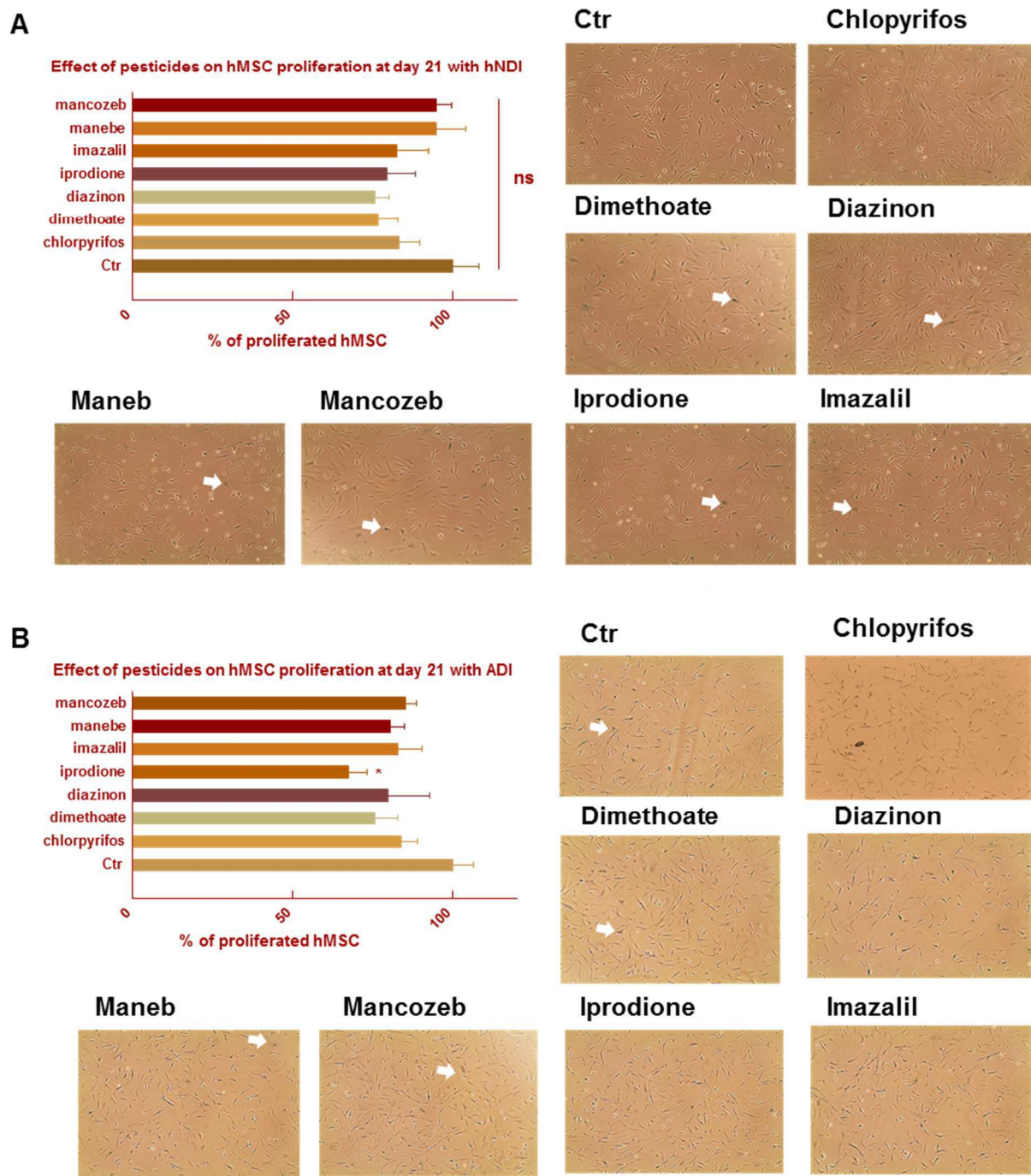
15 2-http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/4038.pdf

16 3- <https://www.anses.fr/fr/system/files/AUT2013sa0138.pdf>
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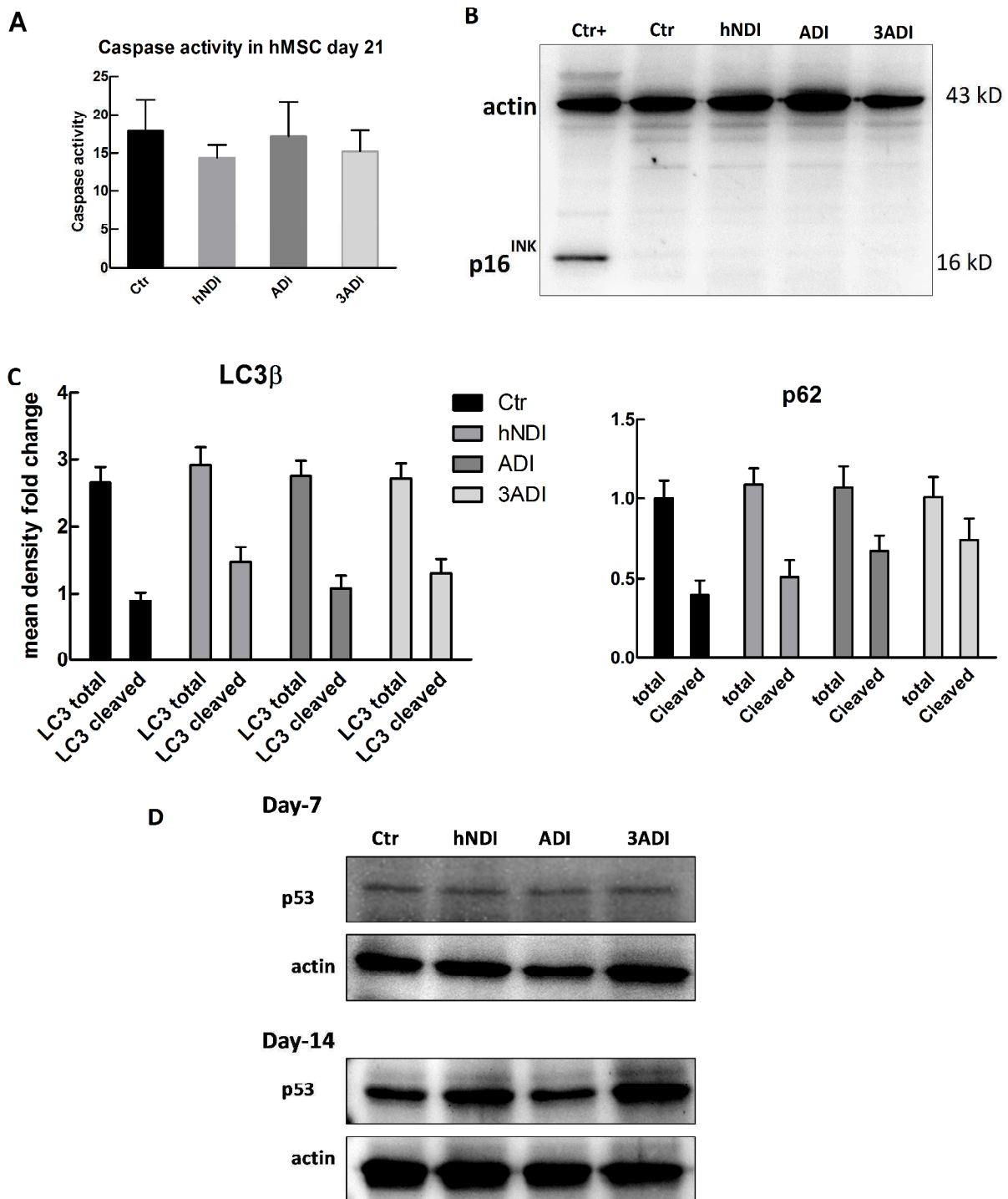
FIGURE S2**A****B****Fig. S2**

(A) MSC proliferation in the absence or in the presence of pesticide mixture for 21 days. MSC were seeded at 2000 cells/cm² 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 Mancozeb individually 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

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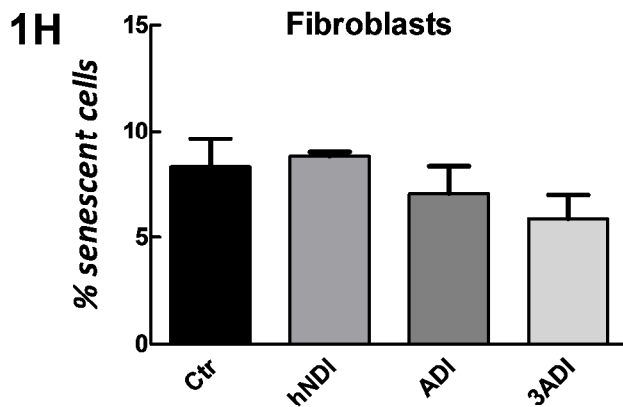
irradiation at 15 gy. **(C)** Semi-quantification of LC3 β cleavage and p62/SQSTM1 degradation after 21-day exposure to pesticide mixture (n=3). **(D)** p53 expression, analyzed by immunoblot at day 7 and 14 after pesticide mixture-treatment (n=3).

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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

2B MSC + Pifithrin α (10 μ M) from day 7

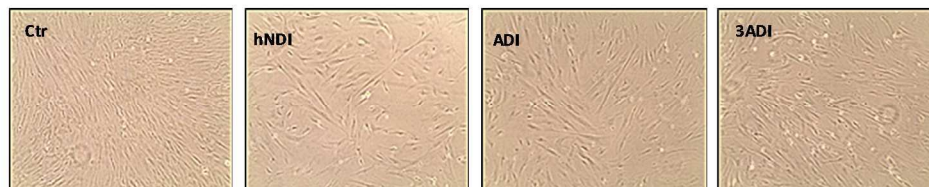


Fig.S5,

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 μ M from day 7) during the 21-day exposure to pesticide mixture.

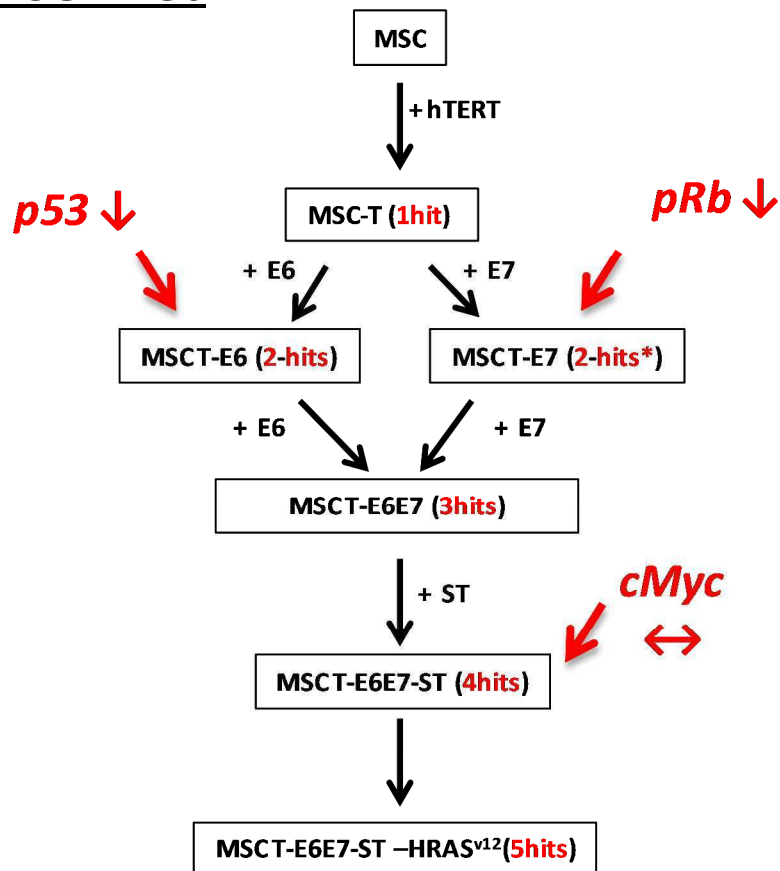
FIGURE S6

Fig.S6

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

MSC were modified and transformed by a stepwise retroviral transduction as follows

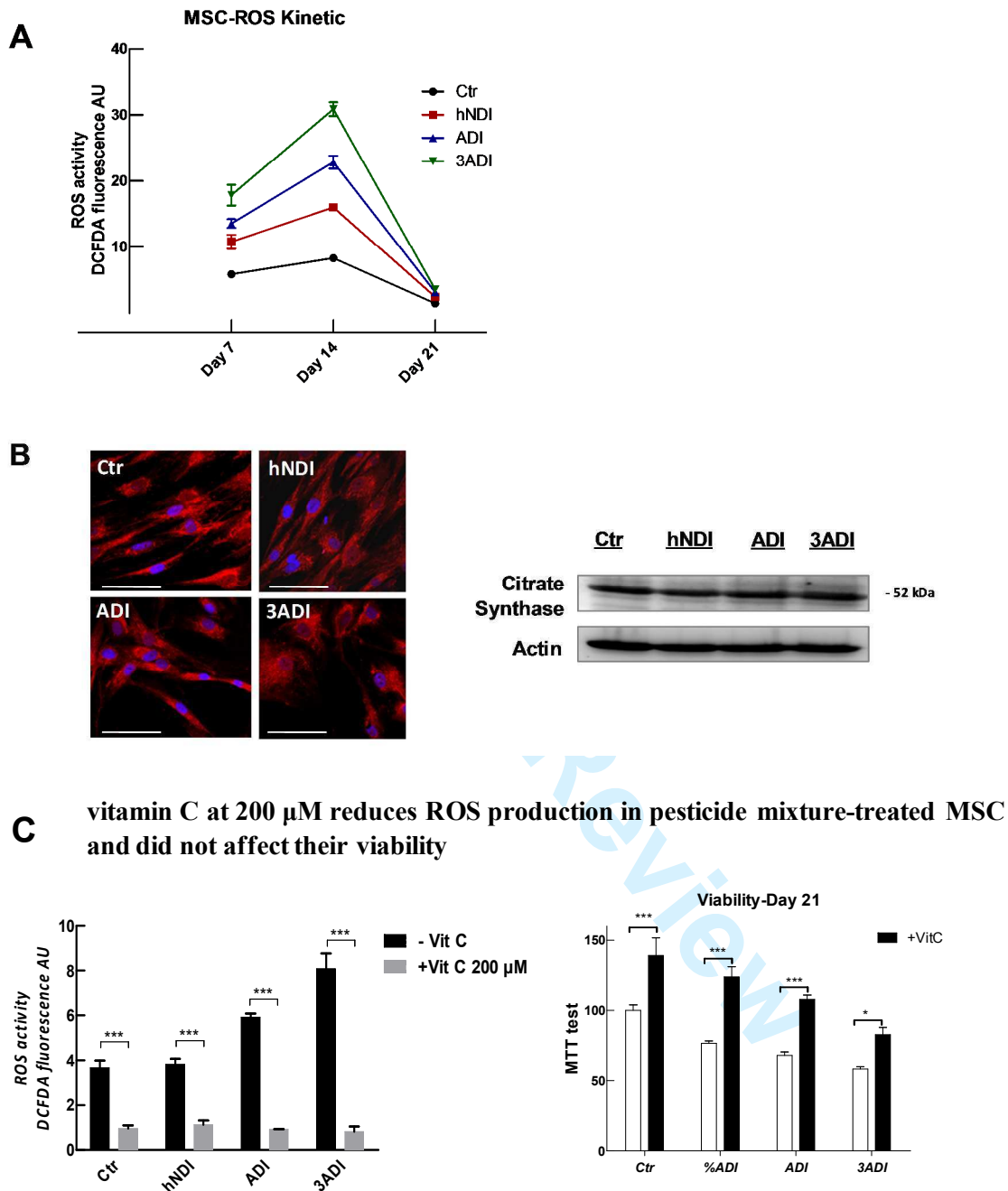
-The catalytic subunit of human telomerase (hTERT) (tMSC-1hit), prolonging lifespan

-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

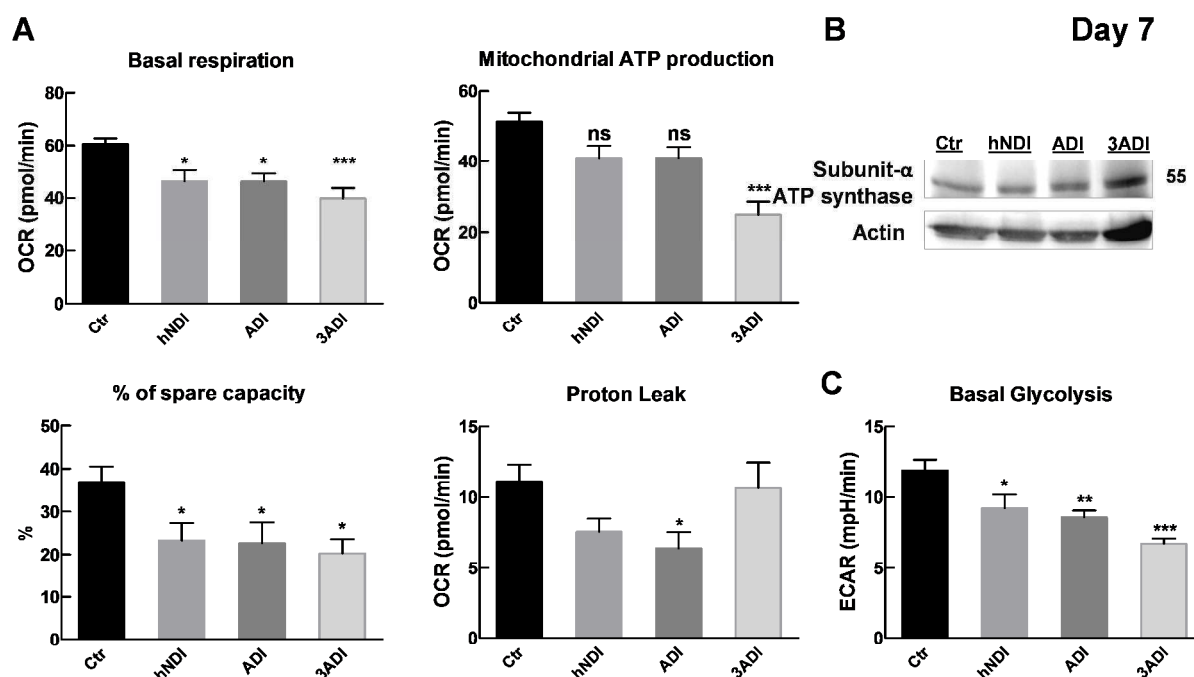
-tMSC- 5hits, + Oncogenic allele of H-Ras (H-Ras^{V12}), H-Ras^{V12} provides acquisition of a constitutive mitogenic signal.

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 CMH₂-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

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3 was analyzed in MSC after exposure to pesticide mixture for 21 days by immunoblot. (C)
4 MSC were treated with vitamin C (200 μ M) in the presence or in the absence of pesticide
5 mixture during 21 days. Cell viability was measured at day 21 using MTT assay
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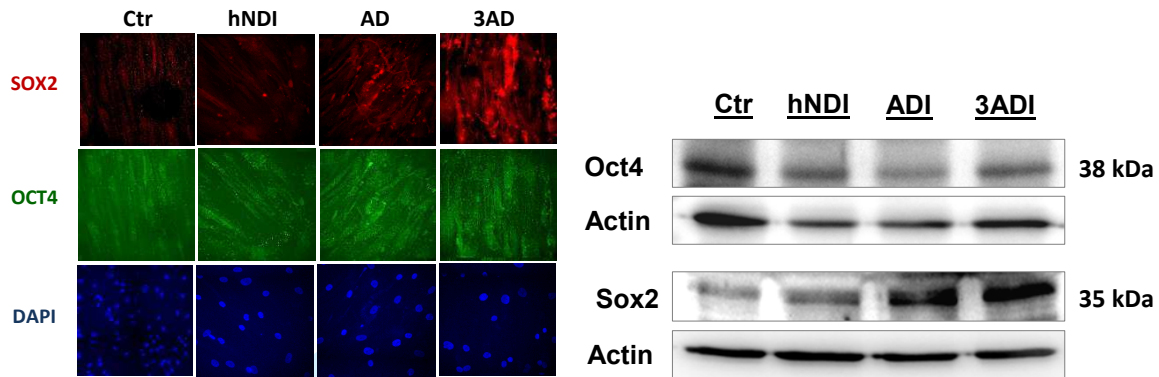
FIGURE S8**Fig.S8**

A, Mitochondrial respiration was assessed using the Seahorse XF24 Flux Analyzer. After 7 days of exposure to pesticide mixture, cells (2×10^4) 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. ($n=5$, $*p<0.05$, $**p<0.01$).

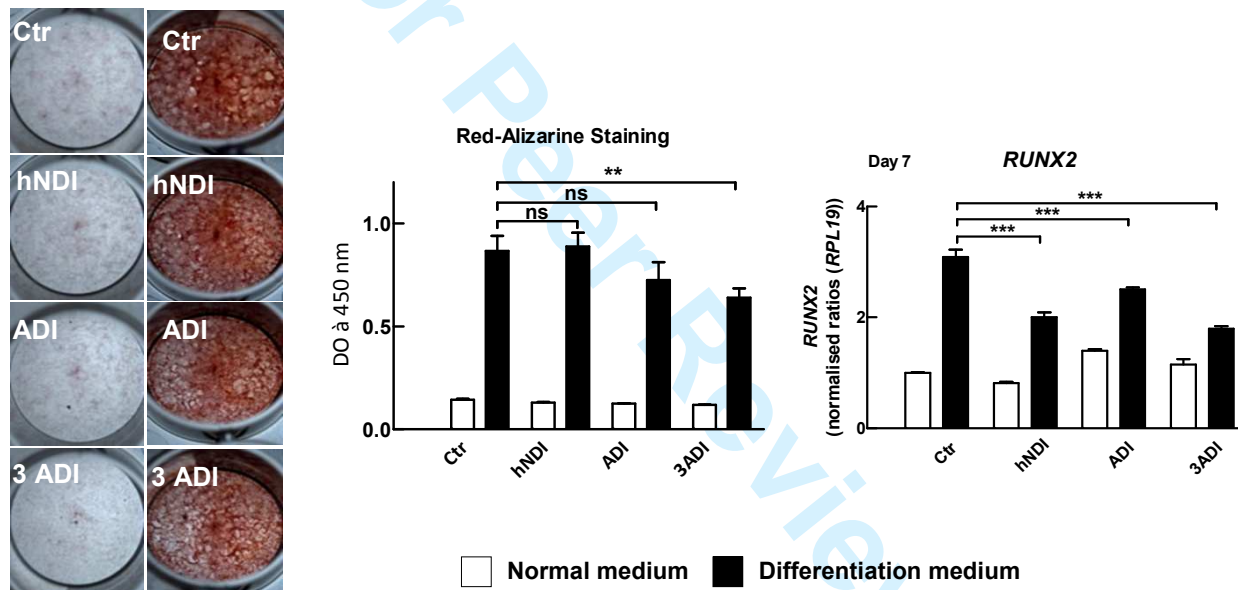
Mitochondrial respiration was significantly affected after 7-day pesticide mixture treatment with a reduction in cellular basal respiration regardless of the dose. This reduction was not associated with less mitochondrial ATP generation in presence of hNDI and ADI but decreased by 50% with 3ADI (oxygen consumption rate $OCR=51.37 \pm 2.49$ 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 $20\% \pm 4$ the spare mitochondrial capacity was preserved versus an average of $35\% \pm 3$ 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

A



B



C

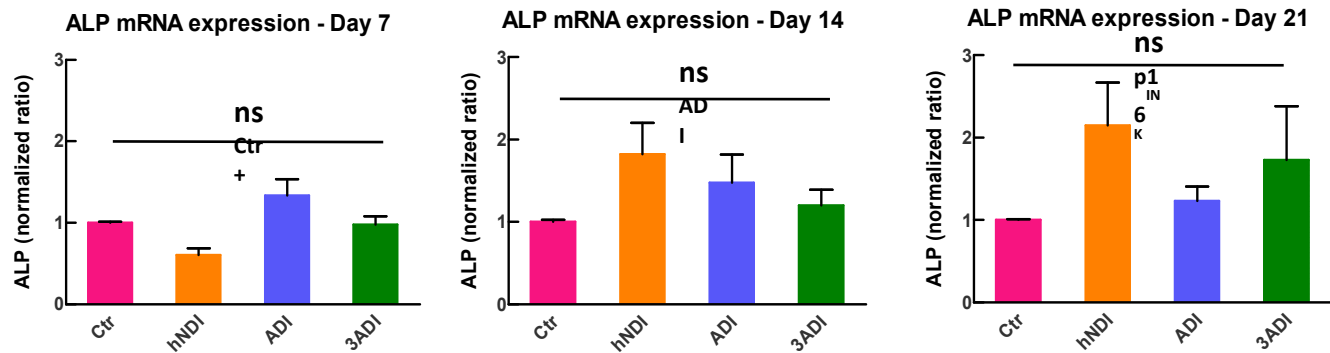


Fig.S9

(A) Expression of stemness markers Sox2 and Oct4 in MSC was evaluated by immune fluorescence (bars 200 μm) and immunoblot 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

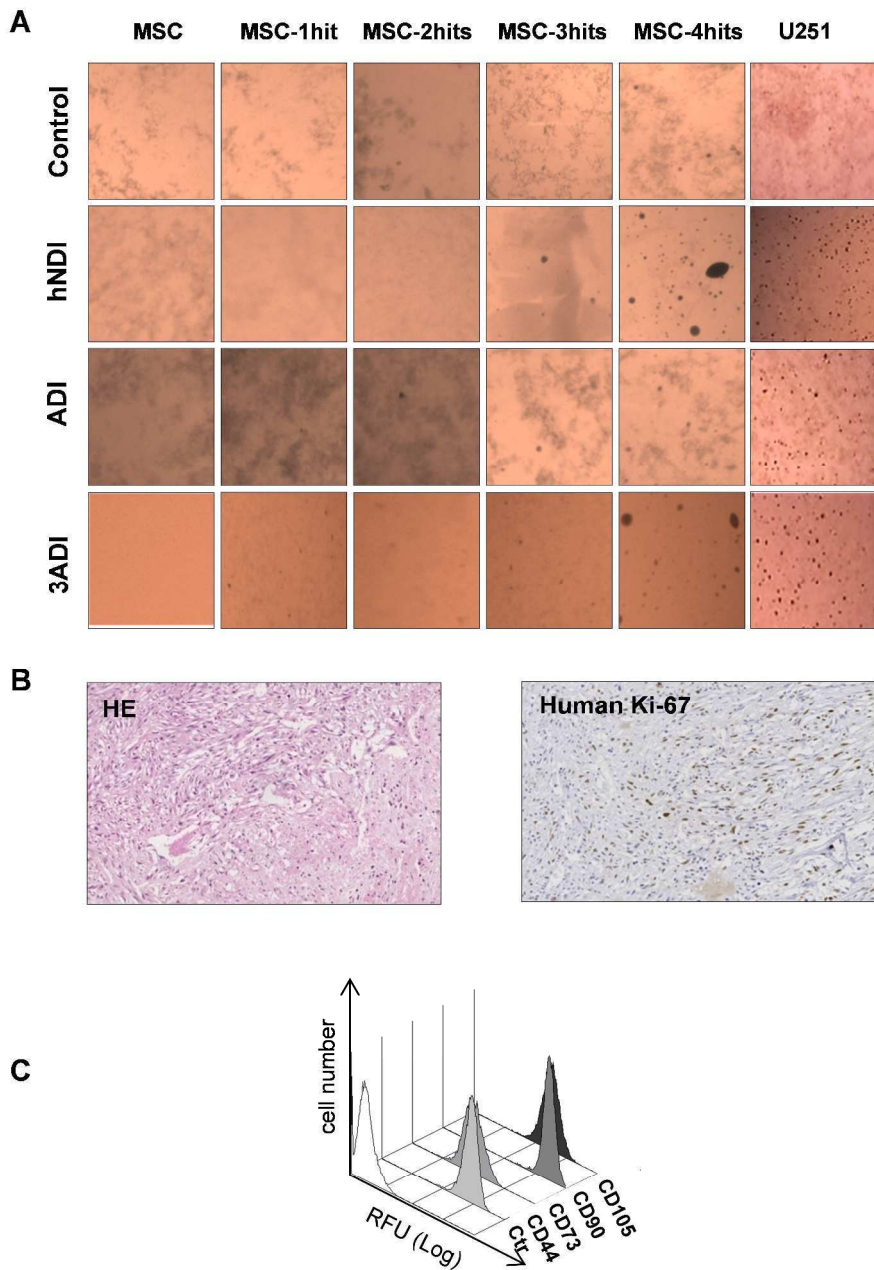


Fig.S10

(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-X1	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 (g/mol)	ADI (mg/kg b.w./d) EFSA 2010	Average exposure according to EFSA 2010			
			CellsExposure concentrations ($\mu\text{mol/l}$)	hNDI (in % ADI)	hNDI	ADI
Chlorpyrifos	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

<i>Cells</i>	<i>Hits</i>	<i>Ctr</i>	<i>hNDI</i>	<i>ADI</i>	<i>3ADI</i>
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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