A complex secretory program orchestrated by the inflammasome controls paracrine senescence

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Oncogene-induced senescence (OIS) is crucial for tumour suppression. Senescent cells implement a complex pro-inflammatory response termed the senescence-associated secretory phenotype (SASP). The SASP reinforces senescence, activates immune surveillance and paradoxically also has pro-tumorigenic properties. Here, we present evidence that the SASP can also induce paracrine senescence in normal cells both in culture and in human and mouse models of OIS \textit{in vivo}. Coupling quantitative proteomics with small-molecule screens, we identified multiple SASP components mediating paracrine senescence, including TGF-\(\beta\) family ligands, VEGF, CCL2 and CCL20. Amongst them, TGF-\(\beta\) ligands play a major role by regulating p15\textsuperscript{INK4b} and p21\textsuperscript{CIP1}. Expression of the SASP is controlled by inflammasome-mediated IL-1 signalling. The inflammasome and IL-1 signalling are tumour suppressive effects\textsuperscript{3,4} but the SASP also mediates important contradictory effects\textsuperscript{8}. Initial studies focused on the pro-tumorigenic properties of the SASP (refs 5–7) but the SASP also mediates important tumour suppressive effects\textsuperscript{8}. Specific components of the SASP such as IGFBP-7, PAI-1, IL-6 and CXCRC2-binding chemokines (such as IL-8 or GRO\(\alpha\)) can reinforce senescence\textsuperscript{9–11}. The SASP also contributes to the surveillance and elimination of senescent cells by the immune system\textsuperscript{12–14}.

It is unclear whether pro-senescent effects can be exerted in a non-cell-autonomous fashion (paracrine) in addition to a cell-autonomous fashion (autocrine) and whether senescence can be transmitted to normal cells. Early experiments where young and old fibroblasts were mixed suggested that senescence was exclusively cell intrinsic\textsuperscript{15,16} although more recently a bystander senescence response has been suggested\textsuperscript{17}. However, although some factors secreted by senescent cells, such as IL-6, reinforce senescence in an intracrine fashion\textsuperscript{9}, others such as IGFBP-7 can exhibit paracrine effects\textsuperscript{11}. In this investigation, we present unequivocal evidence supporting that senescence can be transmitted in a paracrine fashion, and provide insights into the pathways regulating and mediating paracrine senescence.

**RESULTS**

Paracrine transmission of senescence by cells undergoing OIS

To understand whether cells undergoing OIS can transmit senescence in a non-cell-autonomous manner, we established co-cultures of senescent and normal human IMR90 fibroblasts. Cells were distinguished by expressing an mCherry fluorescent marker (Cherry) in the normal IMR90 cells. The Cherry-positive and -negative populations were monitored using high content analysis microscopy.
Cells undergoing OIS can induce paracrine arrest of normal cells. (a) Co-culture with cells undergoing OIS induces the arrest of normal IMR90 cells. Normal IMR90 human fibroblasts expressing the fluorescent marker mCherry (IMR90 Cherry) were mixed with IMR90 ER:RAS cells. Where indicated, 200 nM 4OHT was added to activate ER:RAS. Growth curves represent the number of IMR90 ER:RAS (left) or IMR90 Cherry cells (right) present in the co-cultures. Data are for a representative experiment of \( n = 2 \) independent experiments for days 0–7 and mean ± s.d. of \( n = 3 \) independent experiments for day 8. The source data for two independent experiments and statistics for day 8 (Student’s t-test) are provided in Supplementary Table S8. (b) Co-culture with IMR90 MEK:ER cells induces the arrest of normal IMR90 cells. The percentage of BrdU+ cells for each population in the co-cultures 4 days after 4OHT induction is shown. Data are for a representative experiment. The source data for two independent experiments are provided in Supplementary Table S8. (c) IMR90 ER:RAS or IMR90 ER cells were co-cultured with normal IMR90 Cherry fibroblasts. BrdU incorporation at day 7 shows that co-culture with IMR90 ER:RAS but not with IMR90 ER induces the arrest of normal IMR90 Cherry cells (centre). Data are for a representative experiment. The source data for two independent experiments are provided in Supplementary Table S8. Representative images are shown (right). Scale bar, 30 µm. IF, immunofluorescence staining. (d) Normal HMEC or IMR90 cells suffer growth arrest when co-cultured with HMEC cells undergoing OIS. HMEC TERT (centre) or IMR90 (right) cells expressing Cherry as a fluorescent marker were co-cultured with HMEC TERT ER:RAS or HMEC-TERT vector cells in the presence of 100 nM 4OHT. Growth curves showing growth arrest of HMEC TERT Cherry (centre) or IMR90 Cherry cells (right) are shown. Data are for a representative experiment.

(Fig. 1a and Supplementary Fig. S1a,b). We used IMR90 ER:RAS cells expressing a chimaeric fusion protein that activates on treatment with 4-hydroxytamoxifen (4OHT). RAS activation triggers growth arrest, and induces senescence effectors and the SASP (Supplementary Fig. S2a). IMR90-ER:RAS cells co-cultured with normal IMR90 Cherry cells also undergo arrest on 4OHT treatment (Fig. 1a, middle).

Importantly, normal IMR90 Cherry cells also stopped proliferating when co-cultured with cells undergoing OIS, which suggested a non-cell-autonomous (paracrine) transmission of senescence (Fig. 1a, right panel). Controls confirmed that Cherry-positive cells did not express ER:RAS (Supplementary Fig. S2b). Normal IMR90 Cherry cells also underwent arrest when co-cultured with IMR90 MEK:ER cells (Fig. 1b),...
Figure 2 Paracrine senescence is a stable arrest mediated by soluble factors. (a) Quantification of the percentage of IMR90 ER:RAS (centre) and IMR90 Cherry cells (right) incorporating BrdU during the course of 7 days after activation with 4OHT in co-cultures. Data are for a representative experiment. The source data for two independent experiments are provided in Supplementary Table S8. (b) Kinetics of the production of secreted factors during OIS. Conditioned medium (CM) from IMR90 ER:RAS cells was collected and used to detect CXCL1 (left) or IL-8 (right) levels by enzyme-linked immunosorbent assay. Data are for a representative experiment. The source data for two independent experiments and statistics for day 4 are shown. (Student’s t-test) Fibroblast CM induced the arrest of normal IMR90 fibroblasts (Fig. 1d right and Supplementary Fig. S2d), suggesting that paracrine senescence can be transmitted between different cell types. These results clearly show that senescence can be transmitted.

Paracrine senescence is a stable arrest mediated by secreted factors
We noted that normal cells arrested with a slight delay when compared with cells undergoing OIS in co-cultures (Fig. 2a). We reasoned that senescence can be transmitted between different cell types. These results clearly show that senescence can be transmitted.
that this delay could be attributed to a paracrine response, as the induction of SASP components (CXCL1, IL-8, CCL-20, activin A or VEGFc) occurred as early as 2–3 days after RAS activation (Fig. 2b and Supplementary Fig. S3a).

To investigate whether soluble factors mediate paracrine senescence, we used Transwell inserts that ensure physical separation of the cells (Fig. 2c and Supplementary Fig. S3b). IMR90 cells at the bottom exhibited a senescent morphology and became arrested when co-cultured in the presence of senescent cells in the top chamber (Supplementary Fig. S3b). Next, we co-cultured normal cells and IMR90 ER:RAS for 7 days using Transwells. At that point we split the IMR90 cells, and cultured them alone for 14 another days (Fig. 2c). Cells that have undergone paracrine senescence continued exhibiting features of senescence, suggesting that the transmitted phenotype is stable (Fig. 2c). Analysis using quantitative PCR with reverse transcription (qRT–PCR) discarded cross-contamination between cells or transmission of the RAS oncogene in the Transwell experiments (Supplementary Fig. S3c).

To confirm that factors secreted by senescent cells were sufficient to induce paracrine senescence, we exposed normal IMR90 cells to conditioned medium (CM) from IMR90 cells expressing active forms of RAS, RAF or MEK. Whereas cells exposed to CM from control cells grew normally, those treated with CM from senescent cultures showed reduced BrdU incorporation with a higher percentage staining positive for SA-β-galactosidase (SA-β-Gal; Fig. 2d and Supplementary Fig. S3d). The cell arrest persisted after withdrawal of the CM (Supplementary Fig. S3e). Similar results were observed on mouse embryo fibroblasts (Supplementary Fig. S3f).

As paracrine senescence seemed dependent on soluble factors, we reasoned that its effects should be spatially restricted. To investigate this, we seeded a cluster of IMR90 ER:RAS cells surrounded by normal IMR90 mCherry cells (Fig. 2e). Normal IMR90 Cherry cells in close proximity to the IMR90 ER:RAS cluster (in 3 optical fields, equivalent to up to 1 mm) showed reduced BrdU incorporation with a higher percentage staining positive for SA-β-galactosidase (SA-β-Gal; Fig. 2d and Supplementary Fig. S3d). The cell arrest persisted after withdrawal of the CM (Supplementary Fig. S3e). Similar results were observed on mouse embryo fibroblasts (Supplementary Fig. S3f).

Next we co-cultured IMR90 Cherry and IMR90 ER:RAS cells and monitored the expression of senescence markers and effectors by high content analysis using thoroughly validated antibodies (Supplementary Fig. S1c–e). On activation of RAS, IMR90 ER:RAS cells in the co-cultures exhibited high DNA and oxidative damage and upregulated expression of the CDKIs, p16INK4a and p21CIP1 and of IL-8, a component of the SASP (Fig. 3a, top centre). Normal cells (IMR90 Cherry) in the co-cultures also showed increased levels of oxidative and DNA damage and activation of p16INK4a, p21CIP1 and IL-8 expression, suggesting a full transmission of senescence (Fig. 3a, top right). A similar induction of senescent features was observed in normal cells co-cultured with IMR90 MEK:ER cells undergoing OIS (Supplementary Fig. S4a).

Global gene expression exposed a high correlation between IMR90 cells undergoing OIS and paracrine senescence (Fig. 3b and Supplementary Fig. S4b). Unsupervised hierarchical clustering grouped OIS and paracrine senescence (Fig. 3c) and a transcriptional signature associated with senescence was significantly upregulated during paracrine senescence (Fig. 3d). In addition, qRT–PCR confirmed that CDK inhibitors and the SASP were induced during paracrine senescence (Supplementary Fig. S4c and Table S1).

To understand whether paracrine senescence is commonly associated with senescence, we compared paracrine senescence and OIS induced by MEK activation, observing a significant overlap of upregulated genes (Supplementary Fig. S4d). Moreover, we derived a paracrine senescence signature and used gene set enrichment analysis (GSEA) to interrogate its association with senescence transcriptomes. Different human and mouse cell types undergoing replicative senescence or oncogene- or stress-induced senescence exhibited an enrichment of the paracrine senescence signature (Fig. 3e and Supplementary Fig. S4e,f). Amongst them, HMEC cells undergoing OIS expressed key SASP components, suggesting a similar implementation of paracrine senescence (Supplementary Fig. S4g). The paracrine senescence signature was also associated with murine pancreatic intraepithelial neoplasias (PanIN) and human SSAs (Fig. 3e and Supplementary Fig. S4f), lesions that are both enriched in senescent cells. To examine whether paracrine senescence depends on the same genetic networks as OIS, we knocked down key effectors of senescence in IMR90 cells and either exposed them to CM of senescent cells or co-cultured them with cells undergoing OIS. These experiments revealed that the paracrine senescence arrest depends on the activation of p16INK4a, p21CIP1 and p53 (Fig. 3f and Supplementary Fig. S4h).

Multiple components of the SASP mediate paracrine senescence

We next catalogued the secretome of cells undergoing OIS using stable isotope labelling of amino acids in culture (SILAC, Fig. 4a). Unbiased quantitative proteomics offered several advantages for breadth of coverage and its ability to detect changes in protein expression not apparent from gene expression profiling (Fig. 4b). Amongst the top hits identified were chemokines, TGF-β family ligands or VEGF (Fig. 4c and Supplementary Table S2). To identify which factors mediate paracrine senescence, we compiled a collection of 78 chemical compounds targeting their receptors or key downstream pathways activated by the SASP (Supplementary Table S3). Normal IMR90 cells treated with the drug library were exposed to CM from cells undergoing OIS and BrdU incorporation was assessed 48 h later. Out of the compounds partially impairing the arrest, several targeted the VEGFR2/FLT3, TGFBR1 and CCR2 receptors (Fig. 4d and Supplementary Table S3). These compounds were confirmed to inhibit paracrine senescence in a dose-dependent manner, except the CCR2 inhibitor that exhibited a biphasic effect (Fig. 4e). By using RNA-mediated interference (RNAi) to knock down the expression of CCR2 or the TGF-β receptors ALK4, ALK5 (also known as TGFBR1) and ALK7, we confirmed their role (Fig. 4f,g). These results suggest that multiple factors secreted by senescent cells mediate paracrine senescence.

The TGF-β pathway mediates paracrine senescence

We also interrogated the chemical compounds library for their ability to influence RAS-induced senescence (Supplementary Fig. S5a and...
Figure 3 Paracrine senescence depends on the p16<sup>INK4a</sup>/Rb and p53/p21<sup>CIP1</sup> tumour suppressor networks. (a) IMR90 Cherry cells were co-cultured with IMR90 ER:RAS cells in the presence or absence of 200 nM 4OHT. Seven days after 4OHT treatment, cells were subjected to immunofluorescence (IF) staining. The percentage of IMR90 ER:RAS (top, centre) or IMR90 Cherry cells (top, right) positive for each of the markers is shown. Data are for a representative experiment. The source data for two independent experiments are provided in Supplementary Table S8. Representative pictures are shown at the bottom. Scale bar, 30 µm. (b) mRNA expression profiling of IMR90, IMR90 ER:RAS or IMR90 cells cultured in Transwells together with IMR90 vector or IMR90 ER:RAS cells for 7 days in the presence of 200 nM 4OHT and 0.5% FBS. The plot shows the correlation between cells undergoing OIS and paracrine senescence. (c) Hierarchical clustering of genes changing more than twofold, centred in a cluster that defines the equivalence between OIS and paracrine senescence. (d) GSEA of a signature associated with senescence<sup>18</sup> in IMR90 cells undergoing paracrine senescence. (e) A signature derived from IMR90 cells undergoing paracrine senescence is found enriched in mouse PanIN (ref. 44) and human SSAs (ref. 36). NES, normalized enrichment score; FDR, false discovery rate. (f) Paracrine senescence is dependent on the p53/p21<sup>CIP1</sup> and p16<sup>INK4a</sup> pathways. IMR90 cells were transfected with the indicated siRNAs. The next day, CM from IMR90 ER:RAS or IMR90 vector cells was added. The proliferation of IMR90 ER:RAS cells was assessed by BrdU incorporation 2 days after CM addition (right). Data are mean ± s.d., n = 3 independent experiments. The source data and statistics (Student’s t-test) are provided in Supplementary Table S8. IMR90 cells transfected with the different siRNAs were subjected to immunofluorescence staining as a control for knockdown efficiency (centre).
Figure 4 Multiple components of the SASP are involved in paracrine senescence. (a) Diagram summarizing the proteomics approach. (b) Comparison between mRNA and protein expression for the secretome of cells undergoing OIS. Overall Pearson correlation is 0.64. A lower correlation was observed for proteins induced more than fourfold (red line, Pearson correlation = 0.15). This suggests post-transcriptional upregulation of SASP components (for example, MMP7, IGFBP5, IGFBP6, THBS1, THBS2 and IL6ST). (c) Plot of 2 forward and reverse SILAC experiments. Significant changes in at least 2/3 experiments are coloured. (d) Screening for compounds inhibiting paracrine senescence. IMR90 fibroblasts were grown in the indicated CM in the presence of a collection of 78 drugs. Two days later, BrdU incorporation was measured. –, IMR90 treated with DMSO and grown in CM of IMR90 Vector +4OHT; +, IMR90 treated with DMSO and grown in CM of IMR90 ER:RAS +4OHT. The grey area represents an arbitrary cutoff of 120%, the value of BrdU in the no-drug control (+). Inhibitors over the cutoff targeting VEGFR2 and/or FLT3 (orange), CCR2 (green) or TGFBR1 (brown) are marked. Data are mean ± s.d., n = 3 independent screen plates. (e) IMR90 cells cultured for 2 days with the indicated CM and drugs (concentrations 10 µM to 10 nM). Proliferation was evaluated by BrdU incorporation. Graph shows one representative experiment out of two independent experiments. (f) Infected IMR90 cells were treated with CM and growth was evaluated by CV (top). Data are for one representative experiment out of two independent experiments. Immunofluorescence (IF) staining against CCR2 is shown as a control for the efficiency of the shRNAs used (bottom). Scale bar, 10 µm. (g) Knockdown of receptors of the TGF-β family partially rescue paracrine senescence. IMR90 cells infected with the indicated vectors were treated with CM of senescent or control cells and senescence evaluated 10–14 days after by CV (top left) and SA-β-Gal staining (bottom left). Knockdown efficiency was measured by qRT–PCR (right). Data are for one representative experiment out of two independent experiments. The source data for two independent experiments are provided in Supplementary Table S8. Scale bar, 50 µm.
Table S3). In addition to the compounds identified as affecting paracrine senescence the autocrine senescence screen showed that inhibition of IL1R signalling also prevented OIS (Fig. 5a and Supplementary Fig. S5a). The comparison between both screens suggested that TGFBR1 inhibitors had a more pronounced effect on paracrine rather than on autocrine senescence (Fig. 5a). In fact, although GSEA unveiled an association of TGF-β1 signalling with both OIS and paracrine senescence (Fig. 5b), most TGF-β-dependent genes were more prominently upregulated during paracrine senescence than OIS (Fig. 5c).

TGFBR1-type receptors bind multiple TGF-β family ligands. Although TGF-β1 was also induced, other ligands of the TGF-β
and BMP branches, including BMP6, BMP2, inhibin A and GDF15, were more acutely upregulated during senescence (Fig. 5d and Supplementary Fig. S5b). BMP-like ligands and TGF-β-like ligands signal through activation of different SMAD family members. The phosphorylation of both SMAD2/3 and SMAD1/5 was upregulated in cells undergoing paracrine senescence (Fig. 5e and Supplementary Fig. S5c), corroborating the involvement of both branches of TGF-β signalling in senescence. The effect of BMP2 on senescence has been reported and further confirmed by us (Supplementary Fig. 5d). Moreover, combination of blocking antibodies targeting TGF-β1, activin A (a homodimer of inhibin A) or BMP2 partially rescue the arrest observed during paracrine senescence (Fig. 5e). TGFBRI inhibitors prevented the phosphorylation of SMAD2/3 (Fig. 5f and Supplementary Fig. S5e) and blunted the paracrine senescence arrest (Fig. 5f). These effects correlated with impaired p15\(^{\text{INK4b}}\) and p21\(^{\text{CIP1}}\) induction (Fig. 5f and Supplementary Fig. S5g), consistent with previous observations.

We next investigated whether TGF-β signalling influences senescence in vivo. We used mice bearing a conditional Pdx1-driven activated Kras allele (KRas\(^{G12D}\); ref. 23). KRas\(^{G12D}\) is a potent oncogene in pancreas, but its tumorigenic properties are restrained by its ability to cause OIS, observed in premalignant PanIN lesions\(^{24}\). GSEA showed that TGF-β signalling was associated with these PanIN lesions (Fig. 5g and bottom left). Pdx1\(-\text{cre}\) Kras\(^{G12D}\) mice were crossed with a conditional allele lacking TGF-βR1 (TGF-βR1\(^{β/β}\); ref. 25; Fig. 5g, top left). Lesions observed in Pdx1\(-\text{cre} Kras^{G12D/+}\) mice had characteristics of OIS, with low proliferation and stabilized positive for SA-β-Gal (Fig. 5g). The OIS was attenuated in Pdx1\(-\text{cre} Kras^{G12D/+}\) TGF-βR1\(^{β/β}\) lesions (Fig. 5g). Importantly, Pdx1\(-\text{cre} Kras^{G12D/+}\) TGF-βR1\(^{β/β}\) mice succumbed to a mixture of pancreatic and skin cancer in less than 3 months, but only a subset of Pdx1\(-\text{cre} Kras^{G12D/+}\) animals progress to pancreatic cancer, and with latency of over a year\(^{26,27}\).

**Activation of the inflammasome controls SASP production**

As multiple components of the SASP execute paracrine senescence, we searched for factors coordinating their expression. We screened factors for their ability to induce IL-6 and IL-8, identifying IL-1α as one of the most robust inducers (Supplementary Fig. 56a). IL-1α signalling has been implicated in regulating IL-6 and IL-8 on senescence\(^{28}\). A more thorough analysis identified IL-1α as a potent inducer of multiple SASP components (Fig. 6a,b). Moreover expression of IL-1α caused a SASP-like response phenocopying cells undergoing OIS (Fig. 6c, left). Although cells expressing inhibin A or TGF-β induced some SASP components such as IL-8 or CCL2 (Supplementary Fig. 56b), they did not mimic the SASP (Fig. 6c, centre). Inhibiting TGFBRI did not affect the secretome induced by IL-1α (Fig. 6c, right). In addition, whereas IL-1α inhibition partially prevented induction of IL-8 or CCL2 by TGF-β, the converse was not true (Supplementary Fig. 56b), suggesting that IL-1 has a more prominent role than TGF-β signalling in controlling the SASP.

GSEA showed that IL-1 signalling was associated with paracrine senescence and OIS both in culture and in vivo (Fig. 6d and Supplementary Fig. 56c,d). Molecules involved in IL-1 signalling (such as IRAK family kinases) were also induced during OIS (Fig. 6e and Supplementary Fig. 56e). IL-1α and IL-1β are synthesized as precursors. In particular, pro-IL-1β is inactive until processed by the inflammasome, a multi-protein complex comprising caspase 1 and several adapter molecules\(^{29,30}\). Cells undergoing OIS secreted the processed, mature forms of both IL-1α and IL-1β, suggesting inflammasome activation (Fig. 6f). Indeed, IMR90 cells undergoing OIS exhibited caspase-1 activity (Fig. 6g, left). The inflammasome was also activated during OIS in vivo in braf\(^{P50V}\) -driven murine S\(\alpha\)SAs (ref. 31) and in Kras\(^{G12D}\)-driven PanIN lesions (Fig. 6g, centre and right). Protein levels of inflammasome components such as caspase 1, ASC (also known as PYCARD) and NLRP3 (but not NLRP1) were upregulated during OIS (Fig. 6h and Supplementary Fig. 56f) without noticeable changes to their messenger RNA levels (Supplementary Fig. 56g), suggesting protein stabilization. Cells undergoing OIS secreted NLRP3 and caspase 1, a characteristic of caspase-1-dependent unconventional secretion\(^{32}\) (Supplementary Fig. 56h). Finally, treatment with caspase-1 or IL-1R inhibitors, (Fig. 6i and Supplementary Fig. 56i) but not TGFBRI inhibitors (Supplementary Fig. 56j), blunted the expression of SASP components during OIS, suggesting that activation of the inflammasome was a cause rather than an effect of the SASP.

**The inflammasome and IL-1 signalling reinforce senescence**

Ectopic IL-1α expression arrested IMR90 cells (Fig. 7a) causing senescence (Fig. 7b) accompanied by increased oxidative and DNA damage, and induction of p53 and p21\(^{\text{CIP1}}\) (Fig. 7c and Supplementary Fig. 57a). Conversely, knockdown of the IL-1 receptor or inflammasome components partially prevented OIS (Fig. 7d). These results were extended using short interfering RNAs (siRNAs) targeting downstream adapter molecules of the IRAK family (Supplementary Fig. 57b–d). Treatment with caspase-1 inhibitors partially prevented the induction of p21\(^{\text{CIP1}}\) and the cell cycle arrest observed during OIS (Fig. 7e), but inhibition of IL-1R signalling using either short hairpin RNAs (shRNAs; Fig. 7f and Supplementary Fig. 57e) or blocking antibodies targeting IL-1α and IL-1β (Supplementary Fig. 57f) also affected paracrine senescence.

We next took advantage of a model where OIS is induced in mouse livers through stable, transposon-mediated transfer of oncogenic Nras (Nras\(^{G12V}\)). This model was used to show that senescent hepatocytes undergo immune-mediated clearance (designated senescence surveillance), important for tumour suppression\(^{12}\). Using this model, we tested whether blockade of IL-1 and other SASP components affected hepatocyte senescence. After injection with the Nras\(^{G12V}\) transposons, mice were treated daily with the indicated compounds for 12 days (Fig. 7g). Although the percentage of cells positive for Nras expression was similar 6 days after transduction (Supplementary Fig. 57g), 12 days after injection the percentage was higher in mice treated with either IL-1R inhibitor or a combination of drugs (targeting IL-1R, VEGFR2, CCR2 and TGFBRI), reflecting reduced clearance of senescent hepatocytes by the immune system and/or senescence inhibition. To analyse the effect on senescence we measured p16\(^{ink4a}\) and p21\(^{\text{CIP1}}\) levels, observing that treatment with IL-1R inhibitor or the drug combination reduced the percentage of senescent hepatocytes (Fig. 7h,i and Supplementary Fig. 57h). Treatment with IL-1R inhibitor also resulted in a significant percentage of Nras+ cells proliferating (Supplementary Fig. 57i). The effect of inhibiting IL-1 was further confirmed using IL-1α-neutralizing antibodies (Supplementary Fig. 57j). Overall, the above results highlight the relevance of IL-1 signalling and SASP regulation for senescence in vivo.
Figure 6 The inflammasome regulates the senescence secretome. (a,b) IMR90 cells were infected with a vector that expresses IL-1α or a control and immunofluorescence staining of the indicated SASP components was performed. Scale bar, 30 μm. (b) Quantification of a. (c) IL-1α activates a SAMP-like response. IMR90 cells were infected with retroviruses expressing RASG12V, IL-1α or inhibin A. Where indicated 4 μM TGFB1 inhibitor II was used. CM was used to probe chemokine and cytokine antibody arrays. (d) GSEA of the IL1R pathway in the gene expression profile of IMR90 cells undergoing OIS (left) and mouse PanIN (right). FDR, false discovery rate; NES, normalized enrichment score. (e) Western blotting (WB) with antibodies against inflammasome components. A control of IMR90 ER:RAS cells cultured in the presence or absence of 200 nM 4OHT were subjected to immunofluorescence staining with antibodies against inflammasome components. A control of IMR90 ER:RAS cells + 4OHT with primary antibody is shown in the lower row. Scale bar, 10 μm. (f) IMR90 ER:RAS or IMR90 vector cells were cultured in the presence of 200 nM 4OHT and 0.5% FBS. Pro, precursor form; mat, mature form. (g) Activation of the inflammasome during OIS, IMR90 ER:RAS cells (left) and murine models of SSA (centre) and PanIN (right) exhibit increased caspase-1 activity. Data are mean ± s.e.m.; n = 4, 3, 6 and 8 different samples for control (GI tract), SSA, control (pancreas) and PanIN, respectively. (h) IMR90 ER:RAS cells cultured in the presence or absence of 200 nM 4OHT were subjected to immunofluorescence staining with antibodies against inflammasome components. A control of IMR90 ER:RAS cells + 4OHT with primary antibody is shown in the lower row. Scale bar, 30 μm. (i) IMR90 ER:RAS or IMR90 vector cells were cultured in the presence of 200 nM 4OHT and 0.5% FBS for 7 days with 10 μM caspase-1 inhibitor or 20 μM IL1R antagonist. After that time, cells were processed and qRT–PCR against different SASP components was performed. Data are mean ± s.e.m., n = 3 independent experiments. Data from this experiment are also presented in Supplementary Fig. S6j. Uncropped images of blots are shown in Supplementary Fig. S9.

Paracrine senescence is observed in mouse and human models of OIS in vivo

To investigate whether paracrine senescence occurs in pathophysiologically relevant conditions in vivo, mouse and human models of OIS were analysed. First we revisited the model where OIS is induced in mouse hepatocytes by NraG12V (ref. 12). The senescent hepatocytes are found surrounded by clusters of immune cells12 (Fig. 8a). We observed that many cells in these clusters stained positive for senescence markers (Fig. 8a). To confirm these findings, we used keratin 5 (K5–Sos Egrf–/–) transgenic mice35. These mice develop papillomas with characteristics of OIS as confirmed by staining for p16INK4a and p21CIP1 within the basal and suprabasal layers of the papilloma (Fig. 8b). Although there were no senescent cells in the tissue close to normal skin, we observed senescent cells present in the K5– tissue adjacent to the senescent papillomas (Fig. 8b and Supplementary Fig. S8a). Examination of their morphological features
identified fibroblasts, lymphocytes and plasma cells, but not cells with epithelial characteristics, amongst the senescent cells in the vicinity of papillomas (Supplementary Fig. S8b).

Finally, we looked for evidence of paracrine senescence during human tumorigenesis studying SSA. Colon SSAs are mainly driven by activating BRAF mutations that trigger OIS (refs 31,34,35). Epithelial tissue from human SSAs, but not normal colonic crypts, was positive for senescence markers such as p21\(^{\text{Cip1}}\) and negative for proliferation markers such as Ki67 (Fig. 8c). SASP components such as CCL2 and IL6 were induced in SSAs (Fig. 8d and Supplementary Fig. S8c).

**Figure 7** IL-1 signalling regulates senescence. (a) IMR90 cells were infected as indicated and proliferation was measured by CV. (b) SA-β-Gal staining. Scale bar, 50 μm. (c) Immunofluorescence staining using the indicated antibodies. Data are for a representative experiment. Source data for two independent experiments are provided in Supplementary Table S8. (d) IMR90 ER:RAS cells were infected as indicated and cell growth was analysed by CV (right); qRT–PCR showing knockdown efficiency (left). Data are for one representative experiment out of two independent experiments. (e) IMR90 ER:RAS cells were treated as indicated. BrdU incorporation and p21\(^{\text{Cip1}}\) expression were measured. Data are mean±s.d., n = 3 independent experiments. P was calculated using Student’s t-test. For BrdU, IL1R inh, p53, p21\(^{\text{Cip1}}\), p16\(^{\text{INK4a}}\) and negative for proliferation markers such as Ki67 (Fig. 8c). SASP components such as CCL2 and IL-6 were induced in SSAs (Fig. 8d and Supplementary Fig. S8c).
Paracrine senescence is observed in mouse and human models of OIS in vivo. (a) Paracrine senescence in the liver. Immunohistochemistry (IHC) images showing senescent hepatocytes surrounded by clusters of immune cells. H, hepatocyte. Immune cells positive for p21CIP1 (left) and p16INK4a (right) are marked by asterisks. Scale bar, 50 µm. (b) Stromal cells in the vicinity of papillomas in the skin of K5–Sos_Egfrwa2/− mice show the presence of cells positive for p21CIP1 and p16INK4a. Arrows show senescent cells in the K5− population. Immunofluorescence (IF) staining for p16INK4a (pictures, top) and p21CIP1 (pictures, bottom) is shown in red. K5 is shown in green. Nuclei are shown in blue. Merged pictures are presented. A close up is shown to the right, and a wider view in the left. Bars are 10 µm. Graph showing the quantification of p16INK4a (top, centre) and p21CIP1 (top right) nuclear levels in cells in the epidermis (K5+) or dermis (K5−) are shown (left). For p21: wt K5+, wt K5−, n = 102; wt K5−, n = 108; Sos K5+, n = 460; Sos K5−, n = 536. For p16: wt K5+, n = 100; wt K5−, n = 105; Sos K5+, n = 597; Sos K5−, n = 939. n = number of cells. Arrows point to stromal senescent cells. Scale bars, 10 µm. (c–e) Stromal cells in the vicinity of human SSA present high levels of p21CIP1 expression. (c) Epithelial cells in the crypts from SSAs are positive for activated BRAF and p21CIP1, but negative for Ki67. Close ups correspond to images shown in the right panel. Scale bar, 25 µm in left panel and 100 µm in the right panel. (d) Immunohistochemistry showing representative staining of CCL2 in normal colon and SSA. 6/11 samples showed higher CCL2 expression in SSA than normal colon, whereas 2/11 have a higher pattern of expression in normal colon than SSA. Scale bar, 100 µm. (e) The percentage of stromal p21CIP1+ cells close to the normal colon or SSA is significantly different. Box represents the first and third quartiles and the line inside shows the median. Whiskers extend upwards and downwards to the highest or lowest observation. Unpaired t-test (P = 0.03, n = 10 samples per condition).

Analysis of expression data also showed the upregulation of IL-1β and other SASP components in SSA (Supplementary Fig. S8d). Using automated imaging analysis (Supplementary Fig. S8e) we measured a significant increase in p21CIP1+/stromal cells (Fig. 8c,e, P = 0.03) or p21CIP1+/Ki67− stromal cells (P = 4.6 × 10−5) close to SSAs compared with tissue close to normal colonic crypts. These cells had immune or fibroblast morphology (Supplementary Fig. S8f). These data suggest that senescence can be transmitted in a non-cell-autonomous fashion in both mouse and human models of OIS in vivo.

**DISCUSSION**

The SASP can potentiate the tumorigenic properties of cancer cells, recruit the immune system to eliminate premalignant cells, or reinforce senescence. Here we add paracrine senescence to the
repertoire of functions regulated by the SASP. Paracrine senescence could be relevant in different scenarios in vivo. As well as reinforcing the arrest of a cell undergoing OIS, its secretome could induce arrest on the surrounding epithelium, expanding the senescent footprint of the preneoplastic lesion and promoting their immune clearance. Alternatively, cells undergoing OIS could propagate senescence to the surrounding tissue, as suggested here (Fig. 8). Other scenarios are possible: therapy-induced senescence influences chemotherapy; radiation can induce a bystander response, which could be partially explained by paracrine senescence; clearance of senescent cells is also beneficial for age-associated disorders. Therefore, it is worth investigating whether paracrine senescence mediates the deleterious effects of senescent cells on tissue homeostasis.

Not all cells surrounding preneoplastic lesions undergo paracrine senescence. Levels of soluble factors, gradients of their concentration, the susceptibility of different cell types and even whether the cells are dividing or arrested are possible factors influencing which cells undergo paracrine senescence in vivo. The nature of these cells and the functional implications of paracrine senescence remain open questions for future studies. Intriguingly, recent work suggests that tumours have the ability to induce p16\\NKp expression in their surrounding stromal and infiltrated immune cells, similar to what we have observed here.

In this study, we identified multiple SASP factors with previously unrecognized roles in controlling senescence, for example TGF-β family members (activin A and GDF15), VEGF, and the chemokines CCL2 and CCL20. Although multiple components of the SASP amplify the response, at least in part by activating NF-κB (refs 41,42), IL-1α is a key regulator not linked previously with senescence induction. There is a wealth of information on the activation of the inflammasome by infectious agents, but less is known about links with other cellular stresses. The ability of the inflammasome to regulate senescence adds unrecognized roles in controlling senescence, for example TGF-β family members (activin A and GDF15), VEGF, and the chemokines CCL2 and CCL20. Although multiple components of the SASP amplify the response, at least in part by activating NF-κB (refs 41,42), IL-1α is a key regulator not linked previously with senescence induction.

In conclusion, cells undergoing OIS can transmit paracrine senescence to their neighbours. Here, we identified that paracrine senescence is a complex response regulated by the inflammasome and IL-1 signalling. Understanding the significance and regulation of paracrine senescence may be the first step towards manipulating it for therapeutic benefit.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Chemical compounds and neutralizing antibodies. 4-hydroxytamoxifen (4OHT) was from Sigma. 5- Ac-YVAD-CMK and z-YVAD-FMK (caspase-1 inhibitor IL and VI) were from Calbiochem. Chemical compounds and concentrations used are summarized in Supplementary Table S4. Neutralizing antibodies targeting BMP2, TGF-β1, activin A, IL-1α and IL-1β (R&D) were used at 10 µg ml−1.

Cell culture. HEK293T and IMR90 cells were obtained from the ATCC. Cells were maintained as described previously54. HMEC-hTert cells were cultured in mammary epithelial cell growth media (PromoCell). Co-culture of IMR90 and HMEC was carried out in mammary epithelial cell growth media (PromoCell) supplemented with 10% FBS. We used 0.02 µm Anopore cell culture inserts (Nunc-Thermo) for Transwell co-culture experiments. Cell numbers and cell viability were determined using Guava Viacount reagent (Millipore) and a Guava cytometer (Millipore).

Retroviral and lentiviral infection. Retroviral and lentiviral infection were performed as previously described55,56.

Plasmids. pRETRO1SUPER (pRS) plasmids expressing shRNas targeting pTqRGEAS, p53 or p2157,58 and pBARE-Ras57,58 pLNC-ER:RAS and pLNC-MEKER have been described previously54. MSCV-puro-based retroplasm encoding IL-1α was generated by cloning its cDNA from pCMV6 IL-1α (Origene). pGIPZ-based shRNA vectors targeting ALK4, ALK5, ALK7, IL1R1, CASP1, ASC and TP53 were from Sigma.

BrdU incorporation, growth curves, colony formation assays and senescence-associated β-Gal staining. These methods have been described elsewhere56,59.

Conditioned medium. The indicated cells (2 × 105) were seeded in a 10 cm dish and incubated for 7 days with 200 nM 4OHT in DMEM with 0.5% FBS. After incubation, the conditioned medium (CM) was collected, centrifuged at 5,000g and filtered through a 0.2 µm pore filter. CM was mixed with DMEM 40% FBS in a proportion of 3 to 1 to generate CM containing 10% FBS.

Transfection of siRNAs. IMR90 fibroblasts were reverse-transfected with 30 nM siRNA using a 3.5% solution of HiPerFect transfection reagent (QIAGEN). AllStars scrambled siRNA served as negative controls. For a list of siRNAs, see Supplementary Table S5.

Gene expression analysis. RT–qPCR was performed as described previously52. Primer sets and TaqMan Gene Expression Assays (Applied Biosystems) used are listed in Supplementary Table S6.

Microarray analysis. For global gene expression studies, cRNA was hybridized to Human Gene 1.0 ST arrays (Affymetrix). Microarray data processing and analysis was carried out at EMBL. Data were normalized using Robust Multichip Average (RMA) and differentially expressed genes were identified using Linear Models for Microarrays (LIMMA). A cutoff of a Benjamini–Hochberg false discovery rate <0.05 was used to identify significant genes. All analyses were carried out in R (v2.13.0).

Gene set enrichment analysis. We used gene set enrichment analysis (GSEA; v2.07) to examine the association between gene sets and gene expression. We ranked vectors targeting ALK4, ALK5, ALK7, IL1R1, CASP1, ASC and TP53 were from Sigma.

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Gene expression analysis. RT–qPCR was performed as described previously52. Primer sets and TaqMan Gene Expression Assays (Applied Biosystems) used are listed in Supplementary Table S6.
(clone VE1) was performed on an automated immunostainer (Ventana BenchMark XT, Ventana Medical Systems) as previously described53. The settings included pretreatment with cell conditioner 1 for 60 min, incubation with undiluted VE1 hybridoma supernatant at 37°C for 32 min and signal enhancement with the Ventana amplification kit (catalogue number 760-080). For Ki67 (clone MB-1, Dako, 1:400) and p21WAF1/Cip1 (clone SX118, DAKO, 1:25), antigens were retrieved using alkaline buffer (pH 9, Dako). The Ki67 and p21WAF1/Cip1 staining procedures were performed using the Techmate 500+ automated staining system (Dako) with the avidin–biotin complex method. p21+ and Ki67+ nuclei in the tumour stroma were counted per area using virtual microscopy (Spectrum Version 11.0.0.725, Image scope v11.0.2.725, Aperio Technologies, Vista). For statistical evaluation, the p21 to Ki67 ratio was determined and compared using the non-parametrical Wilcoxon rank sum test.

Statistical data analysis. Significance levels were denoted as: *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001. Sources for statistical data are provided in Supplementary Table S8.

Accession numbers. Microarray data have been deposited at the Gene Expression Omnibus under the accession number GSE41318.

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Figure S1. High Content Analysis (HCA) to study OIS and paracrine senescence. (a) IMR90 ER:RAS and IMR90 Cherry cells were imaged and the Cherry nuclear intensity of a population of more than 1,000 cells of each is represented here. An intensity of 150 (marked by the blue line) was chosen as a cutoff to discriminate between Cherry positive and negative cells. Note that the intensity scales of the two graphs are different. (b) InCell Analyzer software was used to generate overlay images that discriminated between Cherry positive and negative populations (left) and BrdU positive and negative populations (right) in co-cultures of IMR90 ER:RAS and IMR90 Cherry cells. (c-e) Validation of the different antibodies used for High Content Analysis in this study. Either shRNA or siRNA was used to confirm the specificity of the antibodies. Either constitutive expression of RAS (c), addition of RAS CM (d) or activation of ER:RAS in IMR90 ER:RAS cells (e) was used to induce the different factors. Scale bar, 30 μm in (c) and 10 μm in (d) and (e).
Figure S2 Cells undergoing OIS can transmit senescence to normal cells. 
Related to Fig. 1. (a) IMR90 ER:RAS cells to study OIS. IMR90 ER:RAS and IMR90 Cherry cells were grown with or without 200 nM 4OHT for 7 days and subjected to IF. Data is a representative experiment. The source data for 2 independent experiments are provided in Supplementary Table S8. Representative pictures (bottom) and HCA quantification (top) are shown. Scale bar, 30 μm. (b) IF against ER was performed in co-cultures of IMR90 ER:RAS and IMR90 Cherry cells. Less than 5% of cells scored as IMR90 Cherry expressed the ER:RAS fusion. 4OHT increases levels of ER:RAS, hence the lack of ER:RAS expression in IMR90 ER:RAS cells without 4OHT. Data are mean ± s.d., n = 3 independent experiments. Representative pictures are shown (right). Scale bar, 30 μm. (c) Characterization of OIS in IMR90 MEK:ER cells. IMR90 MEK:ER cells grown for 5 days were subjected to IF. Representative images (left) and HCA quantification (right) is shown. Data is a representative experiment. The source data for 2 independent experiments are provided in Supplementary Table S8. Scale bar, 30 μm. (d) IMR90-Cherry cells arrest when cultured with IMR90 ER:RAS. Cells were co-cultured and growth curves for the IMR90 Cherry cells (right) and IMR90 ER or IMR90 ER:RAS cells (left) in the co-cultures are shown. Data is a representative experiment of 2 independent experiments. Source data are provided in Supplementary Table S8. (e) Growth curve of IMR90 cells derived from a single experiment. Cells at passage 12-15 were used in this paper. (f-g) HMEC-Tert Cherry and IMR90 Cherry cells co-cultured with HMEC ER:RAS in the presence of 100 nM 4OHT stop proliferating. (f) Representative pictures and quantification of HMEC-Tert Cherry cells incorporating BrdU when co-cultured with HMEC ER:RAS or HMEC vector cells. Data is a representative experiment. Source data for 2 independent experiments are provided in Supplementary Table S8. Scale bar, 30 μm. (g) Percentage of IMR90 Cherry cells incorporating BrdU when co-cultured with HMEC cells. Data is a representative experiment. The source data for n = 2 is provided in Supplementary Table S8.
Figure S3 Secreted factors mediate the paracrine senescence arrest. Related to Fig. 2. (a) Kinetics of the production of secreted factors during OIS. Conditioned media from IMR90 ER:RAS cells was collected and used to detect the levels of CCL20 (left), Activin A (centre) or VEGF-C (right) by ELISA. Data is a representative experiment. The source data for 2 independent experiments are provided in Supplementary Table S8. (b) Transwell experiments. Normal IMR90 cells (in the bottom of the transwell) were co-cultured with the indicated cells (IMR90 vector or IMR90 ER:RAS cells at the top), separated by a 0.02 μm Anopore membrane in the presence of 200 nM 4OHT during 7 days. Representative microphotographs are shown (right). Scale bar, 50 μm. (c) RT-qPCR specific for the ER:RAS chimera was performed on control IMR90 cells, and on cells grown in the top and bottom of the transwell to discard cross-contamination or transfer of genetic material encoding for ER:RAS in the transwell experiments. Data are one representative experiment out of 2 independent experiments. (d) CM was collected from IMR90 ER:RAS grown 7 days, or IMR90 MEK:ER or IMR90 RAF:ER cells grown 5 days in the presence of 200 nM 4OHT, and added to normal IMR90 cells. SA-β-Galactosidase assays were performed. (e) The paracrine senescence arrest is stable. IMR90 cells were exposed to conditioned media collected from the indicated cells for 5 days. After that time, cells were split and seeded again, and growth curves were performed for 10 additional days. Data are mean ± s.d., n = 3 independent experiments. Student’s t-Test. For ER-Ras +4OHT vs vector +4OHT comparison p=1.31 x 10^{-5}. The source data for statistics are provided in Supplementary Table S8. (f) CM from MEFs infected with RasG12V induces growth arrest in normal MEFs. Growth curve showing the effect of CM from MEFs infected with RasG12V on normal MEFs, as well as different media conditions.
Paracrine senescence depends on the p16\(^{INK4a}\)/Rb and the p53/p21\(^{CIP1}\) tumor suppressor pathways. Related to Fig. 3. (a) IMR90-Cherry cells were co-cultured with IMR90 MEK:ER cells. 4 days after 4OHT treatment, cells were subjected to IF. The percentage of IMR90 MEK:ER or IMR90 Cherry cells positive for each of the markers is shown (left). Representative pictures (right).

(b) mRNA expression profiling was performed on IMR90, IMR90 ER:RAS or IMR90 cells co-cultured in transwell as indicated during 7 days in the presence of 200 nM 4OHT and 0.5 % FBS. Scattered plots show the low correlation between paracrine senescence and IMR90 control (Pearson correlation: 0.49, left) and between cells undergoing OIS and IMR90 cells co-cultured with normal IMR90 cells (Pearson correlation: 0.32, right).

(c) mRNA expression of CDK inhibitors (left) and SASP factors (right) was assessed by RT-PCR on the indicated cells. Data are one representative experiment out of 2 independent experiments.

(d) Venn diagram showing the overlap of genes which increase >2-fold on paracrine senescence and OIS induced by MEK activation Collado et al., 2005. The overlap is significant (p<2.2x10\(^{-16}\)).

(e) Signature from IMR90 cells undergoing paracrine senescence is found enriched in senescent HMEC cells and senescent human hepatic stellate cells. NES, normalized enrichment score; FDR, false discovery rate.

(f) Summary of datasets of senescent cells used in this paper in which the paracrine senescence signature is enriched as determined by GSEA.

(g) mRNA expression of SASP factors was assessed by qRT-PCR on HMEC cells undergoing OIS. Data are one representative experiment out of 2 independent experiments.

(h) IMR90 Cherry cells were reverse transfected with the indicated siRNAs. Next day, IMR90 MEK:ER cells were seeded together with the IMR90 Cherry cells and cultured for 4 additional days in the presence or absence of 200 nM 4OHT. The growth of the IMR90 Cherry cells was analyzed monitoring BrdU incorporation. Data from one representative experiment out of 2 experiments performed.
**Figure S5** TGFβ signaling is a key mediator of paracrine senescence. Related to Fig. 5. (a) Screening for compounds inhibiting senescence in IMR90 ER;RAS cells (‘Autocrine’ drug screening). IMR90 ER:RAS cells were cultured in the presence of a small collection of 78 chemical compounds in the presence or absence of 200 nM 4OHT to induce OIS. The percentage of cells incorporating BrdU was assessed by IF 5 days after 4OHT addition. A gray area represents an arbitrary cut off of 120 % the value of BrdU in the no drug control. -, IMR90 ER:RAS – 4OHT, treated with DMSO; +, IMR90 ER:RAS – 4OHT, treated with DMSO. Chemical compounds over the cut-off limit targeting VEGFR2 and/or FLT3 (orange), CCR2 (green), TGFBR1 (brown) or IL1-R signaling (blue) are marked. Data are mean ± s.d., n = 3 independent screen plates

(b) Induction of members of the TGFβ superfamily during OIS. Data was taken from the microarrays. The mRNAs which level of expression changed significantly are shown in bold. An arbitrary cut-off is set in gray to identify mRNAs induced more than 2-fold. (c) IMR90 cells activate phospho-SMAD1/5 in response to treatment with CM of senescent cells (RAS CM). Scale bar, 10 μm. (d) BMP2 induces a p21CIP1-dependent growth arrest. IMR90 cells were treated with BSA or BMP2 as indicated. 3 days later the levels of BrdU, p16INK4a and p21CIP1 were analyzed by IF. Data is a representative experiment of 2 independent experiments. The source data for 2 independent experiments are provided in Supplementary Table S8. (e) SMAD2 phosphorylation during paracrine senescence. IB showing total and phosphorylated SMAD 2 in cells treated as explained. 3 neutralizing Abs: Abs against TGFβ1, BMP2 and Activin A. (f) IMR90 cells were treated with CM from IMR90 vector or IMR90 ER;RAS cells in the presence of DMSO or TGFBR1 inhibitors Data are mean ± s.d., n = 3 independent experiments. IF to detect BrdU and p21CIP1 were performed. Data of a representative experiment.
**Figure S6** The inflammasome regulates the expression of the senescence secretome. Related to Fig. 6. (a) Multiple SASP components induce IL-8 and IL-6. IMR90 cells were infected with the indicated retroviruses. IL-8 and IL-6 expression was monitored by IF. Data is a representative experiment of 2 independent experiments. Source data for 2 independent experiments are provided in Supplementary Table S8. Scale bar, 30 μm. (b) Regulation of IL-8 and CCL2 by IL-1 and TGFβ. IMR90 cells were infected and treated as indicated. Levels of IL-8 (left) and CCL2 mRNA (right) were analysed by qRT-PCR. Data are one representative experiment out of 2 independent experiments. (c) GSEA of a signature associated with IL1 signalling is associated with senescent human hepatic stellate cells. NES, normalized enrichment score; FDR, false discovery rate. (d) Heatmap of differentially expressed genes on the IL-1R signature activated during OIS and paracrine senescence. (e, f) IMR90 ER:RAS cells were grown with or without 200 nM 4OHT and after 3 days, cells fixed and subjected to IF against IRAK adaptor proteins (e) or NLRP1 (f). Representative images (left) and quantification (right) (e, f). Data from one representative experiment out of 2 experiments performed. Scale bar, 10 μm. (g) Expression of inflammasome components (microarray experiments). mRNA levels are stable or decrease during OIS. (h) IB showing the expression of NLPR3 and Caspase 1 in CM. (i) Cells undergoing OIS present signs of Caspase 1-dependent unconventional secretion. IB of CM from IMR90 vector cells (Vector) or IMR90 ER:RAS cells (RAS). When indicated cells were treated with an IL1R inhibitor (I) or a Caspase 1 inhibitor (C). mat, mature; pro, precursor form. (j) IMR90 ER:RAS or IMR90 vector cells were cultured in the presence of 200 nM 4OHT and 0.5 % FBS during 7 days with 20 μM IL1R antagonist or 10 μM TGFBR1 Inh II. After that time, cells were processed and qRT-PCR against different SASP components performed. Data are mean ± s.d., n = 3 independent experiments. Data relates to Fig 6i. Fig 6i was plotted in a linear scale and S6j in logarithmic scale.
Figure S7 The inflammasome and IL-1 signaling contribute to senescence reinforcement. Related to Fig. 7. (a) IMR90 cells infected as indicated and subjected to IF. Quantification shown in Fig. 7c. Scale bar, 30 μm. (b-d) IMR90 cells were transfected with the indicated siRNAs and target expression measured by qRT-PCR. Data are mean ± s.d., n = 3 independent experiments. (c) IMR90 ER:RAS cells were transfected with the indicated siRNAs and IF was performed 4 days after 4OHT induction. Data relates to Fig 7d. Scale bar, 30 μm. (d) Proliferation of IMR90-ER:RAS cells transfected with siRNAs 4 days post-transfection. Data are mean ± s.d., n = 3 independent experiments. Student’s t-Test. For BrdU, anti-IL1α p = 0.088, anti-IL1β p = 0.99; anti-IL1α + β p = 0.00038. Source data are provided in Supplementary Table S8. (e) Liver cells positive for NRas expression were quantified 6 days after hydrodynamic injection. Data is mean +/- s.e.m. of n=5 mice. Scale bar, 200 μm. (f) Percentages of Nras positive cells, positive or negative for p16INK4a (left) and p21Cip1 (right). Data relates to Fig 7g-i. (f) Inhibition of IL1R allows the proliferation of NRas positive cells. NRas-positive cells positive for Ki67 were measured 12 days after injection. No Ki67 positive cells are seen for transduction of NrasG12V or NrasG12V/D38A transposons vectors as the liver is quiescent. Data is mean +/- s.e.m. of n=5 mice. (j) Neutralizing antibodies targeting IL-1α abrogate senescence and immune surveillance of premalignant hepatocytes. Quantification on liver sections from mice 12 days after injection Data are mean ± s.d., n = 5 mice per condition.
Figure S8 Paracrine senescence occurs in vivo. Related to Fig. 8. (a) Quantification of the cells positive for senescent markers in the vicinity to skin papillomas. Stroma cells in the vicinity to papillomas in the skin of K5-SOS EGFR<sup>wa2/wa</sup> mice show the presence of cells positive for p21<sup>Cip1</sup>, p16<sup>Ink4a</sup> that are not observed in wild type mice. Presented here is the quantification of the percentage of positive cells corresponding to the data presented in Fig 8b. Data are mean ± s.d., from n=3 papillomas or normal skin samples (b) The nature of the stromal cells positive for p16<sup>Ink4a</sup> or p21<sup>Cip1</sup> in the vicinity to papillomas was assessed based in their morphology by an experienced pathologist and classified as of fibroblast origin (noted by an F) or lymphoid origin (noted by an L). Some senescent plasma cells were also observed (not shown). Scale bar, 100 μm. (c) IHC showing representative staining of IL-6 in normal colon and SSA. Scale bar, 100 μm. (d) Expression of SASP components is upregulated in SSA. Represented is the log (SSA/normal colon) of the expression of SASP components as in Carusso et al., 2009<sup>36</sup>. (e) Automated quantification of senescent cells close to crypts in SSAs. An area close to the SSA was randomly chosen (left) and the software scored the cells measuring their relative intensity for a given marker, in this case p21<sup>Cip1</sup> (right). After that the percentage of positive cells in n=10 cases and controls was calculated. Data is represented in Fig 8f. Scale bar, 100 μm. (f) The stromal cells positive for p21<sup>Cip1</sup> in the vicinity to SSA lesions were assessed for origin based in their morphology by an experienced pathologist and classified as of fibroblast origin (noted by an F) or lymphoid origin (noted by an L). Scale bar, 100 μm.
**Figure S9.** Full scans of immunoblots used in figures.
Supplementary Tables Legends

Table S1 Gene expression analysis of paracrine senescence
Table S2 SILAC experiments to define the senescence secretome.
Table S3 Screening for compounds regulating paracrine or autocrine senescence.
Table S4 Chemical compounds used in this study.
Table S5 siRNAs used in this study.
Table S6 Primers and Taqman probes used in this study.
Table S7 Antibodies used in this study.
Table S8 Statistics source data.