Oncogenic mTOR signalling recruits myeloid-derived suppressor cells to promote tumour initiation

Thomas Welte1,2,3,4, Ik Sun Kim1,2,3,5, Lin Tian1,2,3,6, Xia Gao1,2,3, Hai Wang1,2,3, June Li1,2,3, Xue B. Holdman2,3, Jason I. Herschkowitz1,2,3,11, Adam Pond1,2,3, Guorui Xie7, Sarah Kurley7, Tuân Nguyen4, Lan Liao1, Lacey E. Dobrolecki1,2,3, Lan Pang7, Qianxing Mo2,8, Dean P. Edwards2,3,8, Shixia Huang2,3, Li Xin2,3, Jianming Xu2,3, Yi Li1,2,3, Michael T. Lewis1,2,3, Tian Wang7, Thomas F. Westbrook6,9, Jeffrey M. Rosen2,3,12 and Xiang H.-F. Zhang1,2,3,10,12

Myeloid-derived suppressor cells (MDSCs) play critical roles in primary and metastatic cancer progression. MDSC regulation is widely variable even among patients harbouring the same type of malignancy, and the mechanisms governing such heterogeneity are largely unknown. Here, integrating human tumour genomics and syngeneic mammary tumour models, we demonstrate that mTOR signalling in cancer cells dictates a mammary tumour’s ability to stimulate MDSC accumulation through regulating G-CSF. Inhibiting this pathway or its activators (for example, FGFR) impairs tumour progression, which is partially rescued by restoring MDSCs or G-CSF. Tumour-initiating cells (TICs) exhibit elevated G-CSF. MDSCs reciprocally increase TIC frequency through activating Notch in tumour cells, forming a feedforward loop. Analyses of primary breast cancers and patient-derived xenografts corroborate these mechanisms in patients. These findings establish a non-canonical oncogenic role of mTOR signalling in recruiting pro-tumorigenic MDSCs and show how defined cancer subsets may evolve to promote and depend on a distinct immune microenvironment.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population defined as CD11b+Gr1+ cells. They can be roughly divided into granulocytic and monocytic subsets using Ly6G and Ly6C as markers, respectively. Both CD11b+Ly6G+ and CD11b+Ly6C+ cells have immunosuppressive activities, although different mechanisms may be used. The granulocytic subset is more often found expanded in tumour models and is involved in promoting tumour progression1–4, although anti-tumour effects have also been observed5. In the clinic, MDSCs were first identified in the peripheral blood of cancer patients as non-lymphoid haematopoietic suppressor cells6 that have been subsequently shown to increase during progression in many cancer types7,8. Similar to mouse, most human MDSC carry markers of immature myeloid lineage cells and qualify as either granulocytic (CD11b+CD33+CD15+HDLAlow) or monocytic (CD11b+CD14+HDLAlow) subsets3,4,9.

Considerable information has been obtained about the biogenesis and functions of MDSCs. The cytokines responsible for MDSC accumulation include G-CSF10–14, GM-CSF15, IL-1β16,17, IL-618, PGE219, IFN-γ20, IL-421 and VEGF22. The immunosuppressive mechanisms used by MDSCs involve secretion of TGFβ, generation of nitric oxide and reactive oxygen species, and metabolic depletion of L-arginine by arginase 1 (refs 1,2). These activities can blunt cytotoxicity, block proliferation or induce apoptosis of cytotoxic T lymphocytes and natural killer cells. Other MDSC functions include formation of a pre-metastatic niche23, enhancement of tumour invasion24,25 and stimulation of angiogenesis25.

1Lester and Sue Smith Breast Center, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. 2Dan L. Duncan Cancer Center, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. 3Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. 4Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. 5Diana Helis Henry Medical Research Foundation, New Orleans, Louisiana 70130, USA. 6Graduate Program in Integrative Molecular and Biomedical Sciences, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. 7Verna & Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. 8Department of Microbiology & Immunology, The University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas 77555, USA. 9Department of Pathology and Immunology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. 10Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. 11Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. 12Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. 13McNair Medical Institute, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. 14Present address: Cancer Research Center, University of Albany, 1400 Washington Avenue, Albany, New York 12222, USA. 15Correspondence should be addressed to J.M.R. or X.H.-F.Z. (e-mail: jrosen@bcm.edu or xiangz@bcm.edu).

Received 4 February 2016; accepted 11 April 2016; published online 16 May 2016; corrected after print 20 May 2016; DOI: 10.1038/ncb3355
Despite this knowledge, we have a limited understanding of why and how individual tumours vary widely in their propensity to induce MDSCs. Here, we demonstrate that this propensity is determined by an oncogenic signalling pathway and linked to the subpopulation of tumour-initiating cells (TICs).

RESULTS

Inter-tumoral heterogeneity of MDSC infiltration

We examined myeloid cells in a variety of syngeneic mammary tumour models of diverse genetic backgrounds and tumorigenic drivers. MMTV–WNT1, MMTV–WNT1–iFGFR and P53–PTEN double knockout (DKO) are genetically engineered mouse models in the FVB background. MMTV–WNT1 is a widely used model of basal-like tumours. WNT1–iFGFR is a bigenic model based on MMTV–WNT1, in which FGFR signalling can be inducibly activated\(^{26,27}\). The P53–PTEN DKO was generated by conditional deletion of these tumour suppressors using a MMTV-driven Cre. P53N tumour lines initially arose from transplanted P53-null mammary gland tissues in BALB/c mice, and are maintained through mouse-to-mouse orthotopic transplantation. Despite the common loss of P53, P53N lines exhibited remarkable inter-tumoral heterogeneity in genomic copy number, gene expression profiles and TIC frequencies\(^{28}\). The 67NR–4T07–4T1 series are established cell lines derived from a spontaneous tumour in BALB/c mice. Taken together, these reagents provide an unbiased representation of available syngeneic models.

Mammary tumours were generated either by spontaneous tumorigenesis (MMTV–WNT1, WNT1–iFGFR and P53–PTEN DKO), or orthotopic transplantation of primary tumour tissues (P53N series) or cell suspensions (67NR, 4T07, and 4T1). When tumours reached 1 cm\(^3\), we examined myeloid cell infiltration by immunofluorescence staining of S100A8, which was predominantly expressed by CD11b\(^+\)Gr1\(^+\) cells rather than tumour cells (Supplementary Fig. 1b). Significant inter-tumour heterogeneity was discovered (Fig. 1a and Supplementary Fig. 1a).

Quantification of CD11b\(^+\)Gr1\(^+\) cells in dissociated tumours was largely consistent with S100A8 staining (Fig. 1b). The accumulation of CD11b\(^+\)Gr1\(^+\) cells is systemic, as they were also found in peripheral blood and the frequencies closely correlated with the tumour-infiltrating myeloid cells (Fig. 1c,d). Overall, the inter-model variations of CD11b\(^+\)Gr1\(^+\) cells were as high as 50- to 100-fold. In contrast, tumours of the same model exhibited only a 2- to 5-fold variation (Fig. 1b). Thus, the local and systemic accumulation of CD11b\(^+\)Gr1\(^+\) cells seems to be a stable trait of each tumour line.

Most CD11b\(^+\)Gr1\(^+\) cells expressed a high level of Ly6G and a variable level of Ly6C (Fig. 1e), and exhibited a granulocytic morphology (Fig. 1f). Neutrophils isolated from tumour-free mice also express CD11b and Ly6G. However, P53N-C tumour-induced CD11b\(^+\)Ly6G\(^+\) cells differ from normal neutrophils by their heterogeneous expression of Ly6C (Fig. 1e), which typically indicates an immature status\(^{29}\). Co-culture of CD11b\(^+\)Gr1\(^+\) cells with T cells inhibited CD3- and IL-2-induced T-cell proliferation and IFN-\(\gamma\) production (Fig. 1g,h and Supplementary Fig. 1c), whereas normal neutrophils did not exhibit these effects (Fig. 1h and Supplementary Fig. 1c). Moreover, iNOS expression is over 500-fold higher in CD11b\(^+\)Ly6G\(^+\) cells than in normal neutrophils (Fig. 1i). Thus, these CD11b\(^+\)Gr1\(^+\) cells are immunosuppressive and, therefore, MDSCs by definition.

MDSCs can be depleted by administration of a monoclonal antibody against Gr1 (ref. 12). The depletion inhibited orthotopic growth of P53N-C tumours (Fig. 1j). Moreover, treatment following orthotopic tumour resection significantly reduced distant metastasis to lungs (Fig. 1k) demonstrating the tumour-promoting role of MDSCs.

The oncogenic mTOR pathway dictates MDSC accumulation

To identify the determinant of inter-tumoural heterogeneity of MDSCs, we performed reverse-phase protein array (RPPA) analysis of 200 (phos-)proteins covering major signalling pathways (Supplementary Table 1). MDSC accumulation correlated with the AKT–mTOR and the MAPK pathways across the WNT1, WNT1–iFGFR and P53–PTEN DKO models, and only the AKT–mTOR pathway in the P53N series (Fig. 2a), suggesting that the mTOR pathway is a likely driver of MDSC accumulation. This was largely confirmed by western blot analysis in P53N tumours (Fig. 2b) as well as in the 67NR–4T07–4T1 series (Fig. 2c).

Treatment with rapamycin, an mTOR inhibitor, reduced MDSCs in P53N-C tumour-bearing mice (Fig. 2d). Rapamycin may affect non-cancer cells including MDSCs themselves. To verify that the mTOR activity responsible for MDSC accumulation is cancer cell-intrinsic, we employed a short hairpin RNA (shRNA) to deplete Raptor, an essential protein of the mTOR complex 1, in 4T1 cancer cells (Supplementary Fig. 2). This depletion resulted in a significant reduction of MDSCs \textit{in vivo} (Fig. 2e). We also observed an increase of overall T cells and a decrease of PD1\(^+\) T cells (Fig. 2f). These data strongly support that the oncogenic mTOR signalling may mediate this systemic immunosuppression.

To further distinguish cancer cell-autonomous functions of mTOR from its systemic impact through MDSCs, we performed a ‘rescue’ experiment by adoptively transferring MDSCs into rapamycin-treated animals bearing P53N-C tumours (Fig. 2g), or into animals transplanted with 4T1 cells expressing the shRNA against Raptor (Fig. 2h). In both cases, tumour progression was partially restored (Fig. 2g,h). In contrast, normal neutrophils were unable to rescue tumour progression (Fig. 2h). Thus, at least part of the tumour-promoting effects of the mTOR pathway is through MDSC induction.

The mTOR pathway drives expression of G-CSF

We surveyed over 20 cytokines using multiplexed luminex assays in some P53N models. The serum G-CSF concentration was higher in the P53N-C model than in tumour-free or P53N-A. Other cytokines did not exhibit this trend (Fig. 3a). Expression of a constitutively active mTOR mutant\(^{30}\) in P53N-A tumours led to increased expression of G-CSF (Fig. 3b). Conversely, rapamycin treatment on P53N-C cells/tumours resulted in the reduction of G-CSF in conditioned medium or in serum (Fig. 3c,d). For 4T1 cells, either rapamycin treatment or genetic depletion of Raptor also decreased G-CSF expression (Fig. 3e,f). Taken together, these data support that G-CSF is a downstream target of the mTOR pathway in tumour cells.

We asked whether systemic restoration of G-CSF could rescue delayed tumour progression caused by mTOR inhibition.
Figure 1 Inter-tumoral heterogeneity of MDSC infiltration. (a) Representative immunofluorescence staining of S100A8 in the indicated tumours. Green, S100A8; blue, DAPI (nucleus). Scale bar, 100 μm. (b) Flow cytometry quantification of tumour-infiltrating CD11b+Gr1+ cells. Animal numbers (n) in each model are indicated. Five independent experiments were performed with consistent results and one representative experiment is shown. Lower: Ly6G histogram of tumour-free and P53N-C tumour-bearing mice. Upper: Representative data of Ly6G+ cells in P53N-C tumour-bearing mice compared with tumour-free control. Scale bars, 50 μm. Each panel is representative of six mice per group. (c) Relative tumour volume (normalised to transplant size) in P53N-C tumour-bearing mice. Tumour-induced CD11b+Gr1+ cells in peripheral blood. P53N-C versus tumour-free and P53N-B. n=4; P53N-C: n=5. Three independent experiments were performed with consistent results and one representative experiment is shown. Error bars indicate s.e.m. (d) iNOS expression in MDSCs and normal neutrophils by real-time qPCR. Data derived from three and two animals, respectively. (g) Box-whisker plot of iNOS expression in MDSCs and normal neutrophils by real-time qPCR. Data derived from three and two animals, respectively. (h) Pulmonary metastasis. From left: representative IVIS images, visible lesions, and H&E staining of lung metastases in control and anti-Ly6G groups and bioluminescence quantification. n=4 animals per group, selected from larger group to have similar orthotopic tumour sizes. Scale bars, 10 mm, 5 mm, and 50 μm from left to right. In j,k, the experiment was performed twice with similar consistent results. One representative experiment is shown. Error bars indicate s.e.m. P values are determined by two-tailed Student’s t tests. Statistics source data for c,d,k, are provided in Supplementary Table 4.
Figure 2 The mTOR pathway drives tumour-induced MDSC accumulation. 
(a) RPPA profiling in the indicated mammary tumour models. Signalling molecules involved in the mTOR and MAPK pathways have been selected to make heat maps. Each column represents a biological replicate. Each biological replicate is the mean of a technical triplicate. See Supplementary Table 1 for raw data. (b,c) Western blotting with indicated antibodies to validate RPPA results in indicated tumour models. Each lane shows one independent tumour. Unprocessed original scans of blots are shown in Supplementary Fig. 8. (d) MDSC quantification in blood of animals carrying control 4T1 tumours or 4T1 expressing shRNA against the mTOR complex 1 protein Raptor. n = 8 and 24 animals for control and Raptor shRNA groups, respectively. (g) P53N-C tumour growth curves under different treatment conditions. On day 32, the rapamycin-treated group was randomized into two sub-groups as indicated by the dashed line, one of which received transplantation of 4 × 10^6 MDSCs at two time points (indicated by vertical arrows). Tumour size was normalized for each sample relative to tumour size at the onset of differential treatments. n = 6 animals per group. P value calculation: general linear model accounting for repeated measurements at different time points. (h) Tumour growth curves of 4T1 cells expressing Raptor shRNA (n = 24 animals) or scrambled shRNA control (n = 8 animals). On day 15 after tumour transplantation, the Raptor shRNA group was randomized into three groups with or without adoptive transfer of MDSCs or normal neutrophils (NNs; arrows indicate time points of cell transfers). n = 8 per group after randomization. Experiments in e–h were performed twice with similar consistent results. Error bars indicate s.e.m., and P values are calculated by two-tailed Student’s t tests unless otherwise noted.
Figure 3 The oncogenic mTOR pathway drives MDSC accumulation through G-CSF. (a) Bioplex assays of the indicated cytokines in sera. Data are from two tumour-free, two P53N-A-tumour-bearing and three P53N-C-tumour-bearing mice. (b) Quantification of G-CSF expression by real-time qPCR in P53N-A tumour cells expressing a constitutively active mTOR mutant (S2215Y) or wild-type control. (c) Cell viability (by WST-1 assay) and G-CSF quantity (by ELISA) in P53N-C cells as a function of rapamycin concentration. (d) Quantification of serum G-CSF by ELISA in P53N-C-tumour-bearing mice with rapamycin or vehicle treatment. Two experiments were performed with consistent results. One representative experiment with n=5 animals per group is shown. (e) Rapamycin’s effect on 4T1 cell viability and G-CSF expression. (f) ELISA quantification of G-CSF in conditioned medium of 4T1 cells expressing Raptor shRNA, the scrambled shRNA control or empty vector. In b,c,e,f, two independent experiments were conducted each with technical triplicates. One representative experiment is shown. (g) Growth curves of 4T1 cells expressing Raptor shRNA (n=12 animals) or scrambled shRNA (n=6 animals). On day 17 after tumour transplantation, the Raptor shRNA group was randomized into two groups (n=6 animals each), one of which received G-CSF. The P values were determined using general linear models accounting for repeated measurements at different time points. (h) Representative H&E staining and pulmonary metastasis quantification from the experiments in g, day 28 post tumour transplantation. n=5 animals for the first two groups, and n=4 for the third group. Ten fields were randomly chosen from each animal and averaged. P values are calculated by one-tailed Student’s t tests. Scale bars, 200 μm and 50 μm in the upper and lower panels, respectively. (i,j) Tumour volume (i) and lung metastasis (j) of 4T1 cells expressing G-CSF shRNA or vector control (n=8 animals). On day 17, the G-CSF shRNA group was randomized into three groups: untreated (G-CSF shRNA, n=7 animals), treated with MDSCs (G-CSF shRNA + MDSCs, n=7 animals) or treated with G-CSF + Gr1<sup>+</sup> splenocytes of tumour-free mice (G-CSF shRNA + Gr1<sup>+</sup>, n=4 animals). Tumour volumes on day 24 and lung metastases on day 35 are shown. The lung metastasis burden was graded as described in Methods. P values are calculated by two-tailed Student’s t tests. Scale bar, 5 mm. Error bars indicate s.e.m. Statistics source data for c,i,j, provided in Supplementary Table 4.
We administered recombinant G-CSF protein to mice bearing 4T1 tumours expressing the shRNA against Raptor. This treatment restored orthotopic tumour growth and pulmonary metastasis (Fig. 3g,h). Thus, G-CSF functionally mediates at least part of the tumour-promoting functions of mTOR.

The role of G-CSF in MDSC formation and tumour progression has been investigated in previous studies16-19,31. To confirm these effects in our models, we depleted G-CSF either by a neutralizing antibody in the P53N-C model or by shRNA-mediated knockdown in the 4T1 model. These approaches lowered serum G-CSF by 50% or 70%, respectively (Supplementary Fig. 3a,b), resulted in a decrease of MDSCs (Supplementary Fig. 3c,d), and retarded tumour progression and metastasis (Fig. 3i,j). Importantly, this retardation was reversed by adoptive transfer of MDSCs but not splenic Gr1− cells from tumour-free mice (Fig. 3i,j). The depletion of G-CSF did not generate discernible effects on cancer cells in vitro (Supplementary Fig. 3e), arguing against a simple autocrine effect of G-CSF. Taken together, these data further demonstrate that G-CSF-promoted tumour progression is mediated by MDSCs.

Pharmacological inhibition of mTOR may have confounding effects due to its roles in non-cancer cells. Diverse upstream signals may stimulate mTOR activity, some of which are more cancer-specific, such as activation of FGFR and loss of PTEN (Fig. 2a). Indeed, the induction of FGFR signalling in WNT1−iFGFR bigenic tumours led to a sevenfold increase of serum G-CSF (Fig. 4a). 4T1 cells are known to possess autocrine FGFR signalling22. Reanalysis of a published data set revealed that treatment of 4T1 cells by TKI258, a potent FGFR inhibitor, reduces Csf3 expression, the gene encoding G-CSF. Other MDSC-inducing cytokines did not exhibit the same trend (Fig. 4b). Furthermore, inhibition of the upstream FGFR signalling by BGJ398 also reduced G-CSF both in vitro (Fig. 4c) and in vivo (Fig. 4d). Accordingly, this inhibition also led to a significant reduction of MDSCs (Fig. 4e). Simultaneous inhibition of both EGFR and FGFR significantly reduced G-CSF expression in the P53N-C model (Fig. 4f), although targeting FGFR alone was ineffective, suggesting a redundant role of these receptor tyrosine kinases. Thus, G-CSF is a mediator of mTOR-driven MDSC accumulation. Targeting upstream activators of the mTOR pathway such as FGFR, therefore, may represent viable therapeutic strategies to mitigate MDSC-mediated tumour progression, in addition to their direct effects on cancer cells.

Genomic analyses support the role of G-CSF and MDSCs in mTOR-driven immuno-suppression and tumour progression

We examined the Cancer Genome Atlas (TCGA)33 data sets comprised of genomic, transcriptomic and proteomic data of human breast cancers. To assess the G-CSF/MDSC status, we obtained a gene expression signature derived from the haematopoietic cells of human breast cancers. To test human xenografts for their ability to induce MDSCs, a strong connection between mTOR activity and G-CSF expression was observed in a panel of patient-derived xenografts (PDXs)38,40 (Fig. 5e). Specifically, we assayed pS6K(T389) as an indicator of mTOR activity by western blotting, and G-CSF expression by quantitative real-time PCR (Supplementary Fig. 4h). PDXs expressing a higher level of pS6K(T389) and G-CSF also induced more MDSCs in vivo (Fig. 5f). Moreover, treatment with mTOR inhibitors, rapamycin or Torin 1, reduced G-CSF in two of the PDX lines cultured in three-dimensional (3D) suspension medium (Fig. 5g).

Collectively, these data suggest that human tumours with an activated mTOR pathway are more likely to have an increased infiltration of MDSCs, a decreased infiltration of T cells and a worse prognosis.

TICs exhibit enhanced G-CSF production

In addition to inter-tumoral heterogeneity, we also observed an intra-tumoral heterogeneity of G-CSF production. Intracellular staining of G-CSF, pS6K(T389), pS6(S235,S236) and pSTAT3(S727) followed by fluorescence-activated cell sorting (FACS; Supplementary Fig. 5a,b) revealed a correlation between G-CSF and mTOR activity at the single-cell level (Fig. 6a). Among the top 5% of G-CSFhigh cells (>2 × mean) in 4T1 and P53N-C models, approximately 60–80% are CD24highCD29high, a widely used definition of TICs, as opposed to 30% in the entire population (Fig. 6b and Supplementary Fig. 5c,d). We then examined a different set of TIC markers, EpCAM and CD49f. Among P53N-C cells, the top 5% of G-CSFhigh cells fell into two distinguishable populations, both expressing to different degrees EpCAM and CD49f. Together they account for over 80% of G-CSFhigh cells (Fig. 6c). Sca-1 and c-Kit have also been used as TIC markers in the 4T1 model41, and Sca-1highc-Kithigh cells were also enriched in the G-CSFhigh subpopulation of both 4T1 and P53N-C tumours (Fig. 6d). Taken together, these data support the conclusion that G-CSFhigh cells enrich for TICs.

The converse hypothesis that TICs overexpress G-CSF is supported by the elevated G-CSF level in CD24highCD29high cells and Sca-1c-Kit+ subpopulations as indicated by FACS (Supplementary Fig. 5e,f), or by enzyme-linked immunosorbent assay (ELISA; Fig. 6e).
A similar conclusion was also reached using EpCAM and CD49 as markers (Supplementary Fig. 5g,h).

To investigate whether TICs have enhanced ability to induce MDSC differentiation as compared with non-TICs, we treated primary bone marrow cells of naive mice with tumour-cell-conditioned medium. On a per-cell basis, tumour cells grown under a TIC-enriching condition (mammospheres in suspension medium) secreted more G-CSF than under the condition favouring differentiation (2D cultures). Accordingly, tumour-conditioned medium obtained under the TIC-enriching condition induced increased MDSCs (CD11b+Ly6G+Ly6Cdim/SDC). This increase was reversed by G-CSF-neutralizing antibodies (Supplementary Fig. 5i).

To test the above conclusions in human cancer, we analysed a SAGE (serial analysis of gene expression) data set of purified CD44+ and CD24+ cancer cells43. CSF3 transcripts were exclusively found in CD44+ cells but not CD24+ cells, suggesting that cancer cells with TIC-like phenotype overexpress G-CSF (Fig. 6f). CD44+ cells also express a significantly higher level of FGFR1 transcripts (Fig. 6g). To examine whether CD44+ cells may possess increased activity of the FGFR and mTOR pathway, we intersected the CD44+ associated genes43 with gene expression signatures that reflect mTOR inhibition37 by rapamycin or FGFR inhibition by TKI258 (ref. 32), and discovered statistically significant overlaps (Fig. 6h). Finally, we treated human primary bone marrow cells with conditioned medium from PDX cells. The TIC-enriched conditioned medium induced more differentiation of CD11b+CD33+CD15+CD16– cells (Supplementary Fig. 5j), which are known to be immature granulocytic cells9,44. Taken together, these results provide evidence for the clinical relevance of the connection between TICs, G-CSF and MDSCs.
**Figure 5** A G-CSF-responsive gene signature (G-CSF-sig) links the mTOR pathway to MDSC infiltration and immunosuppression in human breast cancer. (a) Heat maps showing the expression of the G-CSF signature as a single score (top), RPPA data for phospho-proteins reflecting the mTOR activity (middle), and TCR pathway components (bottom). The red and blue colors indicate expression higher than the population means by 2 standard deviations at the levels of transcription (red) or genomic copy number (blue). The P values are based on Student’s t-test on the Pearson correlation coefficients among different variables. (b) Gene Set Enrichment Analysis (GSEA) shows the correlation between G-CSF-sig and gene sets up- or downregulated by rapamycin and pp242. NES, normalized enrichment score. q, false discovery rate. P values were computed on the basis of comparison against random simulation as implemented in GSEA. (c) Box-whisker plots of G-CSF-sig scores in different subtypes of breast cancer. (d) Kaplan–Meier curves show distant metastasis-free survival in the EMC-MSK data set divided into low-pS6K(T389) PDXs (BCM2147, HCI11, and BCM4195, see **e**) and high-pS6K(T389) PDXs (HC13 and MC1, see **g**) and are adjusted by Student’s t-test. Error bars indicate s.e.m. P = 9.8 × 10^{-15} based on one-way ANOVA across different PDX lines, and 0.00045 by two-sided Student’s t-test between low-pS6K(T389) PDXs (BCM2147, HCI11 and BCM4195, see **e**) and high-pS6K(T389) PDXs (HC13 and MC1, see **e**). (f) Two PDX lines, MC1 and HCI11, were treated with Torin 1 or rapamycin in 3D suspension cultures. The expression of G-CSF was determined by qPCR. The experiment was repeated twice with similar results. One representative experiment is shown. Upper panel: n=4 biological replicates for each group. Lower panel: n=6, 5 and 5 biological replicates for the three groups, respectively. Error bars indicate s.e.m. P values are calculated by two-tailed Student’s t-tests. Statistics source data for **g,f** are provided in Supplementary Table 4.
MDSCs enhance TIC features through Notch signalling

We asked how MDSCs contribute to tumour initiation. MDSCs have been shown to promote angiogenesis\(^\text{24,45}\). The perturbation of the mTOR–G-CSF axis did not seem to affect tumour angiogenesis, as suggested by immunofluorescence staining of CD31 (Supplementary Fig. 6). Monocytes and macrophages have been shown to enhance...
TIC features. We asked whether granulocytic MDSCs have similar functions. The presence of MDSCs increased the number of mammospheres formed by 4T1 cells (Supplementary Fig. 7a). Moreover, MDSCs also disproportionally increased the number of cancer cells expressing murine mammary TIC markers including CD24, CD29, CD49f, Sca-1 and c-Kit (Supplementary Fig. 7b). Co-culturing with MDSCs stimulated several stemness-related genes in cancer cells, including Nanog, LGR5 and MSI-1 (ref. 47) (Fig. 7a). Cancer cells extracted from 4T1 tumours with G-CSF knockdown exhibited reduced frequencies of CD24-high/CD29-high cells and EpCAM-high/CD49f-high cells (Fig. 7b), and a decreased ability to form mammospheres (Supplementary Fig. 7c). These alterations could be rescued by adoptive MDSC transfer (Fig. 7b), suggesting that MDSCs contribute to the expansion of TICs in vivo. Taken together, these data indicate that MDSCs enhance the tumour initiation.

Direct cell–cell interaction seemed to be critical for this function, as MDSCs separated in the Boyden chambers were unable to enhance the stemness-related genes (Fig. 7a). This points to pathways that require cell juxtaposition and dictate stem or progenitor cell properties. The Notch pathway meets these criteria. Indeed, MDSC co-culturing significantly increased expression of multiple canonical Notch target genes in a contact-dependent fashion (Fig. 7c). To determine whether this increase is restricted to cancer cells, we transduced a Notch reporter into cancer cells, and observed that the reporter activity was enhanced by MDSCs. This enhancement was ablated by co-expression of a dominant-negative (DN) mutant of Rbpj or MAML in cancer cells (Fig. 7d). Consistently, the MDSC-mediated increase of CD49f-EpCAM-high cells was reduced by DN-MAML or DN-Rbpj (Fig. 7e). Normal neutrophils were unable to induce the same effects (Fig. 7e). In human models, co-culturing of MDSCs and MCI or HCl11 cells (isolated from the respective PDX models) significantly increased the proportion of CD44+CD24+ cells (Fig. 7f). This increase was almost completely abolished by a Notch inhibitor, semagacestat (Fig. 7f). Finally, the G-CSF-sig correlates with Notch target genes in the TCGA data set (Fig. 7g). Taken together, these results strongly support that MDSCs enrich TICs through Notch signalling in cancer cells.

To test the impact of mTOR–MDSC cascade on tumour initiation in vivo (Fig. 8a), we transiently treated P53N-C-tumour-bearing mice with rapamycin. A subset of the rapamycin-treated mice also received transplantation of exogenous MDSCs to selectively rescue the MDSC-mediated effects downstream of the mTOR pathway. The tumours were then subjected to tumour initiation assays without further rapamycin or MDSC treatment. As expected, compared with control tumours, rapamycin-treated tumours exhibited a significant decrease of CD29+EpCAM-high/CD49f-high cells (Fig. 8b). Provision of exogenous MDSCs reversed this decrease (Fig. 8b). When transplanted into untreated mice at high cell numbers (5,000 and 1,000 cells per mouse), the rapamycin-treated group exhibited slower tumour progression (Fig. 8c and Supplementary Fig. 7d). At low cell numbers (200 cells and 40 cells per mouse) tumour initiation is significantly delayed in this group (Fig. 8d and Supplementary Fig. 7e). There was also a statistically significant decrease of TIC frequency compared with the other groups (P = 0.016 by extreme limiting dilution analysis). The effects of rapamycin on tumour size, latency and TIC frequency were all partially reversed by concomitant transfer of MDSCs (Fig. 8c,d and Supplementary Fig. 7d,e). These data show that the transient inhibition of mTOR signalling reduced TIC frequency, and, more importantly, this reduction is partially mediated by MDSCs.

**DISCUSSION**

Our results indicate that mammary tumours relying on different oncogenic pathways may also differ in their ability to alter the immune system. Recent genomic studies have suggested enormous heterogeneity of tumour-infiltrating immune cells. However, why and how different tumours evolve to enrich distinct immune cells remains poorly understood. Here, our data demonstrate how a tumour cell-intrinsic oncogenic pathway, the mTOR pathway, determines a tumour’s capacity to accumulate granulocytic MDSCs, providing one partial answer to these questions.

Our data demonstrate that G-CSF is a downstream target gene of the mTOR pathway, and mediates MDSC accumulation. The roles of MDSCs and G-CSF in tumour progression and therapeutic responses have been elucidated. However, the role of mTOR-G-CSF cascade has not been reported, and these results extend the oncogenic functions of the mTOR pathway from a cancer cell-intrinsic to a systemic level. Our data suggest a therapeutic strategy to combine current immunotherapies with mTOR inhibitors, which has already been applied to endocrine-therapy-resistant ER+ tumours in the clinic. A recent study used a PI(3)K inhibitor and demonstrated synergistic effects with immune checkpoint blockade, supporting that inhibition of the PI(3)K–mTOR pathway may help overcome immunosuppression and enhance immunotherapies. A potential caveat of this strategy is that the PI(3)K–mTOR pathway also plays an important role in T cells. As a result, PI(3)K and mTOR inhibitors themselves are expected to be immunosuppressive. Here, our data provided mechanistic insights into potential upstream targets including FGFR and G-CSF. Drugs blocking FGFR or G-CSF may inhibit MDSC accumulation without potentially detrimental effects on the adaptive immune system.

MDSCs enhance TIC features. This is consistent with the previously reported role of MDSCs in ovarian cancer. The mechanism here, however, seems to be distinct—through the Notch pathway in tumour cells. In addition, our data also indicate that TICs enrich the ability to produce G-CSF, and hence contribute more significantly to MDSC accumulation. Taken together, we identified a feedforward loop between TICs and MDSCs, suggesting that the tumorigenic potential may evolve hand in hand with tumour-induced immunosuppression.

Among the variety of tumour models we examined, only a subset resulted in granulocytic MDSC accumulation. Thus, these cells are not universally required, and there may be different immune cells playing distinct roles in other tumour contexts. For instance, another major type of MDSC, monocytic MDSCs, also possess potent immunosuppressive activities. Further studies will be necessary to determine whether monocytic MDSCs are involved in different subsets of breast cancers, and are regulated by different oncogenic pathways.
MDSCs promote breast cancer TIC features. (a) Gene expression of Nanog, Lgr5 and Msi-1 in 4T1 mammosphere cultures with or without MDSCs. 4T1 and MDSCs were co-cultured in direct contact, or separated (0.4 μm pore size, Boyden chamber (B.C.)). Cells were cultured in different wells to form 3–6 technical replicates. Three experiments yielding consistent results were performed. (b) Percentage of CD24<sup>hi</sup>CD29<sup>hi</sup> cells (left) or EpCAM<sup>hi</sup>CD49f<sup>hi</sup> cells (right) in mammary tumours formed by 4T1 cells expressing shRNA against G-CSF or vector control. Some of the mice bearing G-CSF shRNA tumours were subjected to MDSC transplantation (G-CSF shRNA tumours). Data were derived from two independent experiments. (c) MC1 and HCI11 cells were maintained in 3D suspension cultures with or without MDSCs. CD24 and CD44 expression was assessed by FACS. The P values were determined using two-way ANOVA with different co-culturing conditions as one factor (two levels: MDSCs and normal neutrophils (NNs)). Data were derived from two independent experiments. (d) Relative bioluminescence intensity of cancer cells expressing a luciferase Notch reporter, either cultured alone or together with MDSCs (+MDSCs). Dominant-negative RBPJ (DN-RBPJ) or MAML (DN-MAML), or control vector was co-expressed with the Notch reporter in cancer cells. n = 13 wells of cells that were independently set up, transfected with plasmids, and co-cultured with MDSCs/NNs. NS, not significant. (e) Quantification of EpCAM<sup>hi</sup>CD49f<sup>hi</sup> cell frequencies in cancer cells cultured in 3D suspension medium either alone, with MDSCs (+MDSCs) or normal neutrophils (+NNs). DN-RBPJ or DN-MAML, or control vectors were expressed in cancer cells. The P value was determined by two-way ANOVA across various treatment groups with different genetic perturbations as one factor (three levels: control, DN-RBPJ and DN-MAML) and co-culturing conditions as the second factor (three levels: control, +MDSCs and +NNs). Data were derived from two independent experiments. (f) MC1 and HCI11 cells were maintained in 3D suspension cultures with or without MDSCs. CD24 and CD44 expression was assessed by FACS. The P values were determined using two-way ANOVA with different co-culturing conditions as one factor (three levels: control, DN-RBPJ and DN-MAML).
Figure 8 The mTOR–MDSC cascade increases TIC frequency in vivo. (a) Schematics of experiments to test the MDSC-mediated effects of the mTOR pathway on tumorigenesis capacity of P53N-C cells. P53N-C tumours were allowed to grow to about 0.5 cm, before being randomized into three groups: untreated, Rapa (rapamycin 5 mg kg$^{-1}$, three times per week for two weeks), and Rapa + MDSCs (4 × 10$^6$ cells transplanted twice a week for two weeks). After the treatment, tumours were extracted and subjected to the assays shown in b–d. (b) Quantification of frequencies of CD29$^+$EpCAM$^+$CD49$^+$ cells of P53N-C tumours subjected to the treatment shown in a. n = 7, 4 and 4 for the three groups, respectively. Error bars, s.e.m.

(c) Results of tumour initiation assays. Tumour formation after injection of equal numbers of tumour cells was compared. Tumour size on day 17 post orthotopic tumour cell injection (5,000 cells per mouse). n = 5 mice per group. Error bars, s.e.m. (d) Kaplan–Meier curve of tumour-free survival. Low tumour cell number injection (40 cells per mouse) reveals increased latency of tumour cells derived from rapamycin-treated mice. Number of mice with tumours at the end of observation time and total numbers of mice are also indicated for each condition. P values were computed by log-rank test. Statistics source data for b are provided in Supplementary Table 4.

METHODS

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

ACKNOWLEDGEMENTS

We would like to thank Novartis for providing BGJ-398, and H. C. Lo and D. Weiss for helpful input. We also thank the Antibody-Based Proteomics Shared Resource of the Dan L. Duncan Cancer Center supported by Cancer Center Support grant NCI P30CA125123, and A. Welm, Huntsman Cancer Institute, USA for generously providing some of the PDX models. X.H.-F.Z. is supported by NCI CA183878, the Breast Cancer Research Foundation, US Department of Defense DAMD W81XWH-13-1-0195, Susan G. Komen CCR1429845, and McNair Medical Institute. T.Welte is supported by the Helis Foundation. H.W. is supported by US Department of Defense DAMD W81XWH-13-1-0296. Studies with the p53-null tumours were supported by NIH grant CA148761 to J.M.R. and with the WNT1–fGFR tumours by NIH grant CA16303 to J.M.R. RRPA experiments were supported by Cancer Prevention and Research Institute of Texas (CPRIT) Core Facilities Support Award RP120092 to D.P.E. The authors acknowledge the joint participation by Diana Helis Henry Medical Research Foundation through its direct engagement in the continuous active conduct of medical research in conjunction with Baylor College of Medicine.

AUTHOR CONTRIBUTIONS

Conception and design: X.H.-F.Z., J.M.R. and T.Welte. Development of methodology: T.Welte, I.S.K., L.T., X.G., S.H., J.X., T.Wang and Q.M. Acquisition of data: T.Welte, I.S.K., L.T., X.G., H.W., J.L., X.B.H., J.I.H., A.P., G.X., S.K., T.N., L.L., D.P.E., S.H., J.X., Y.L., M.T.L., T.Wang, T.F.W. and L.X. Analysis and interpretation of data: T.Welte, X.H.-F.Z., J.M.R., T.Wang, I.S.K. and Q.M. Writing and review of manuscript: X.H.-F.Z., T.Welte, T.Wang and J.M.R. Study supervision: X.H.-F.Z. and J.M.R.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3355

Reprints and permissions information is available online at www.nature.com/reprints

1. Gabrilovich, D. I. & Nagaraj, S. Myeloid-derived suppressor cells as regulators of the immune system. Nat. Rev. Immunol. 9, 162–174 (2009).
2. Bunt, S. K., Clements, V. K., Hanson, E. M., Sinha, P. & Ostrand-Rosenberg, S. Inflammation enhances myeloid-derived suppressor cell cross-talk by signaling through Toll-like receptor 4. J. Leukoc. Biol. 85, 996–1004 (2009).
3. Gabrilovich, D. I., Ostrand-Rosenberg, S. & Bronte, V. Coordinated regulation of myeloid cells by tumours. Nat. Rev. Immunol. 12, 253–268 (2012).
4. Marvel, D. & Gabrilovich, D. I. Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected. J. Clin. Invest. 125, 3356–3364 (2015).
5. Granot, Z. et al. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. Cancer Cell 20, 300–314 (2011).

6. Strober, S. Natural suppressor (NS) cells, neonatal tolerance, and total lymphoid irradiation: exploring obscure relationships. Annu. Rev. Immunol. 2, 219–237 (1984).

7. Almand, B. et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J. Immunol. 166, 678–689 (2001).

8. Diaz-Montero, C. M. et al. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol. Immunother. 58, 49–59 (2009).

9. Solito, S. et al. Myeloid-derived suppressor cell heterogeneity in human cancers. Ann. NY Acad. Sci. 1319, 47–65 (2014).

10. Cao, Y. et al. BMP4 inhibits breast cancer metastasis by blocking myeloid-derived suppressor cell activity. Cancer Res. 74, 5091–5102 (2014).

11. Donkor, M. K. et al. Mammary tumor heterogeneity in the expansion of myeloid-derived suppressor cells. Int. Immunopharmacol. 9, 937–948 (2009).

12. Kowanzet, M. et al. Granulocyte colony-stimulating factor promotes lung metastasis through mobilization of Ly6G+/Ly6C+ granulocytes. Proc. Natl Acad. Sci. USA 107, 21248–21255 (2010).

13. Shojaii, F. et al. G-CSF-induced myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. Proc. Natl Acad. Sci. USA 106, 6742–6747 (2009).

14. Waigt, J. D., Hu, Q., Miller, A., Liu, S. & Abrams, S. I. Tumor-derived G-CSF facilitates neoplastic growth through a granulocytic myeloid-derived suppressor cell-dependent mechanism. PLoS ONE 6, e27690 (2011).

15. Serafini, P. et al. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. Cancer Res. 64, 6337–6343 (2004).

16. Bunt, S. K., Sinha, P., Clements, V. K., Leips, J., & Ostrand-Rosenberg, S. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. J. Immunol. 176, 284–290 (2006).

17. Song, X. et al. CD11b+Gr-1+ immature myeloid cells mediate suppression of T cells in mice bearing tumors of IL-1β-secreting cells. J. Immunol. 175, 8200–8208 (2005).

18. Bunt, S. K. et al. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. Cancer Res. 67, 10019–10026 (2007).

19. Sinha, P., Clements, V. K., Fulton, A. M. & Ostrand-Rosenberg, S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. Cancer Res. 67, 4507–4513 (2007).

20. Gallina, G. et al. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. J. Clin. Invest. 116, 2777–2790 (2006).

21. Bronte, V. et al. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. J. Immunol. 170, 270–278 (2003).

22. Gabrilovich, D. et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. Blood 92, 4150–4166 (1998).

23. Psaila, B. & Lyden, D. The metastatic niche: adapting the foreign soil. Nat. Rev. Cancer 9, 285–293 (2009).

24. Yang, L. et al. Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. Cancer Cell 13, 23–35 (2008).

25. Du, R. et al. HIF1α induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell 13, 206–220 (2008).

26. Welm, B. E. et al. Inducible dimerization of FGFR1: development of a mouse model to analyzepressive transformation of the mammary gland. J. Cell. Biol. 157, 703–714 (2002).

27. Pond, A. C. et al. Fibroblast growth factor receptor signaling dramatically accelerates tumorigenesis and enhances oncoprotein translation in the mouse mammary tumor virus-Wnt-1 mouse model of breast cancer. Cancer Res. 70, 4868–4879 (2010).

28. Herschkowitz, J. I. et al. Comparative oncogenomics identifies breast tumors enriched in functional tumor-initiating cells. Proc. Natl Acad. Sci. USA 109, 2778–2783 (2012).

29. Li, H., Ji, M., Kllarram, K. D. & Keller, J. R. Repression of Id2 expression by Gfi-1 is required for B-cell and myeloid development. Blood 116, 1060–1069 (2010).

30. Sato, T., Nakashima, A., Guo, L., Coffman, K. & Tamanori, F. Single amino-acid changes that confer constitutive activation of mTOR are discovered in human cancer. Proc. Natl Acad. Sci. USA 111, 11774–11779 (2014).

31. Chi, H. Regulation and function of mTOR signalling in T cell fate decisions. Nat. Rev. Immunol. 12, 325–338 (2012).

32. Dey, J. H. et al. Targeting fibroblast growth factor receptors blocks PI3K/AKT signaling, induces apoptosis, and impairs mammary tumor outgrowth and metastasis. Cancer Res. 70, 4115–4126 (2010).

33. Curtis, C. et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 486, 346–352 (2012).

34. Dey, J. H. et al. Targeting fibroblast growth factor receptors blocks PI3K/AKT signaling, induces apoptosis, and impairs mammary tumor outgrowth and metastasis. Cancer Res. 70, 4115–4126 (2010).

35. Wu, Y. et al. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J. Immunol. Methods 347, 70–78 (2009).

36. Varadarajan, S. et al. Tumor-induced myeloid-derived suppressor cells: mechanisms, functional diversity, and clinical relevance in human oncology. Cancer Immunol. Immunother. 61, 1155–1167 (2012).

37. Yang, L. et al. Expansion of myeloid immune suppressor Gr-1+CD11b+ cells in tumor-bearing hosts directly promotes tumor angiogenesis. Cancer Cell 6, 409–421 (2004).

38. Lu, H. et al. A breast cancer stem cell niche supported by juxtafacial signalling from monocytes and macrophages. Nat. Cell Biol. 16, 1105–1117 (2014).

39. Oskarson, T. et al. Breast cancer cells produce tenasin C as a metastatic niche component to colonize the lungs. Nat. Med. 17, 867–874 (2011).

40. Bray, S. J. Notch signalling: a simple pathway becomes complex. Nat. Rev. Mol. Cell Biol. 7, 678–689 (2006).

41. Yu, X. et al. Notch signaling activation in human embryonic stem cells is required for embryonic, but not trophoblastic, lineage commitment. Cell Stem Cell 2, 461–471 (2008).

42. Bollraki, A. et al. Cross-talk between the Notch and TGF-beta signaling pathways mediated by interaction of the Notch intracellular domain with Smads. J. Cell. Biol. 163, 723–728 (2003).

43. Valdez, J. M. et al. Notch and TGFβ1 form a reciprocal positive regulatory loop that suppresses murine prostate basal stem/progenitor cell activity. Cell Stem Cell 11, 676–688 (2012).

44. Yu, H. & Smyth, G. K. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J. Immunol. Methods 331–342 (2006).

45. Kim, K. et al. Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. Proc. Natl Acad. Sci. USA 111, 11774–11779 (2014).

46. Basei, G. et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N. Engl. J. Med. 366, 520–529 (2012).

47. Kim, K. et al. Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. Proc. Natl Acad. Sci. USA 111, 11774–11779 (2014).

48. Chi, H. Regulation and function of T cell fate decisions. Nat. Rev. Immunol. 12, 325–338 (2012).

49. Cui, T. X. et al. Myeloid-derived suppressor cells enhance stemness of cancer cells by inducing microRNA101 and suppressing the co-repressor CIBP2. Immunity 39, 611–621 (2013).
METHODS

Animal studies and tumour models. All animal experiments were carried out in accordance with a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Female animals of 5–6 weeks are used as the recipients of tumour tissue or cell line transplants. BALB/C and SCID/Beige were used as recipients of syngeneic tumours (P53-null series and 4T1–4TO7–67NR cell line series) or PDxS, respectively. MMTV–Wnt1, MMTV–Wnt1-cfGFR and P53–PTEN DKO mice are in the FVB background.

The mouse breast cancer models were as follows: P53N-A (formerly assigned T11), P53N-B (formerly assigned 222SL) and P53N-C (formerly assigned 2208L) from our P53-null mouse mammary tumour bank described in ref. 28 are on the BALB/c background and were used in syngeneic BALB/c mice unless stated otherwise. Models on the FVB background and maintained in FVB strain of mice: MMTV–Wnt1 (termn Wnt1 for brevity) and MMTV–Wnt1 tumours transduced with inducible cfGFR (termnt Wnt1–cfGFR) have been described elsewhere37. The P53–PTEN DKO was generated by conditional deletion of these tumour suppressors using a MMTV-driven Cre through nipple-injection of adenoaviral Cre into transgenic mice carrying respective Fox alleles of P53 and PTEN.

Breast cancer PDxS were maintained by injecting 1–2 mm size tumour pieces into fat pad-cleared mammary glands of SCID Beige mice. The development of PDx lines was conducted under the Institutional Review Board-approved protocols, and was documented in previous studies38,39. The current study used already-established PDxS that had been de-identified, and therefore, has been granted protocol exemption by the Institutional Review Board of Baylor College of Medicine for not involving human subjects. PDxS used in this study: HC11I (ER+), FGFR3), HC13 (ER-), BC1M3143 (Her2+) and triple-negative subtype M1, BCM2147, BCM4272 and BCM4195, which were obtained from two independent sources38,39.

Cell lines. None of the cell lines used in this study is listed in the database of commonly misidentified cell lines maintained by ICLAC and NCBI Biosample. 4T1, in vivo from F. Tamanoi, Tarbiat Modares University, Iran (Addgene plasmid no. 26037).

Lentivirus transduction of tumour cells. The expression vector for a firefly luciferase–GFP fusion protein (FLUC–PWPD) was packaged into lentivirus in 293T cells with pMD2 and pSpAX2 packaging system. Lentiviral stocks were filtered and concentrated by ultracentrifugation. To label P53N-C tumour cells, tumours were digested into single-cell suspensions by collagenase 3 treatment, and incubated with FLUC–PWPD lentivirus and Polybrene for 24 h under mammosphere assay conditions to preserve tumour characteristics. At 48 h culture period, successfully labelled cells were isolated by FACS sorting of GFP-positive cells and injected into mammary glands of BALB/C mice for tumour development. Tumours that retained GFP and luciferase signal (about 40% of tumours) were collected and comprised the tumour stock used in experiments.

4T1 cells were transduced with G-CSF-specific shRNA, Raptor-specific shRNA or a scramble-control nonspecific shRNA using the lentiviral GIPZ vector system (Thermo Scientific Open Biosystems) that allows puromycin selection to obtain a GFP and luciferase signal (about 40% of tumours) were collected and comprised the tumour stock used in experiments. 4T1 cells were transduced with G-CSF-specific shRNA, Raptor-specific shRNA or a scramble-control nonspecific shRNA using the lentiviral GIPZ vector system (Thermo Scientific Open Biosystems) that allows puromycin selection to obtain a GFP and luciferase signal (about 40% of tumours) were collected and comprised the tumour stock used in experiments.

In vivo treatments. 1–2 mm-size tumour pieces were implanted orthotopically, in the fourth mammary gland on the left side of the animal. When tumour size reached an average of 80 mm3, treatments were initiated as indicated in figure legends. In all animals, the initial implantation was conducted on animals at the age of 5–6 weeks.

BGJ398 was dissolved at 10 mg ml−1 in dimethylsulfoxide (DMSO), and mixed with 10% PEG400, 10% Tween80 and 4% ethanol, dosage was at 5 mg kg−1, daily, 5 d per week.

Rapamycin was administered by intraperitoneal (i.p.) injection (100 μg in 200 μl of 10% PEG400, 10% Tween80 and 4% ethanol, dosage was at 5 mg kg−1, three times per week).

Anti-Ly6G treatments comprised of two i.p. injections per week of 100 μg anti-Ly6G (BioXCell) in PBS. Anti-G-CSF (R&D Systems) was i.p.-injected, daily five times per week, after injection dose. Control animals received an equal amount of isotype-control antibody (BioXCell).

Recombinant human G-CSF (Novoprotein, specific activity: 6 × 104 IU mg−1) was injected subcutaneously in the flank at 1.5 μg per injection in 150 μl PBS every 2 days for the duration indicated in the figures.

Gr1+ cells of tumour-bearing mice (MDScs), or for controls, from tumour-free mice, were enriched using a magnetic cell separation kit (Biotinylated anti-mouse Gr1, BD Pharmingen, Biotin Selection Kit, Stem Cell Technologies) yielding >95% purity. For in vivo transfer, cells were washed in PBS and immediately injected through the retro-orbital route. Two consecutive injections of 4 × 106 cells spaced 5 d were applied.

Tumour and tissue analysis. Tumour length (L) and width (W) were measured with a calliper. Tumour volumes were calculated using the formula L × W2/2. MDScs in tumour sections were identified by immunohistochemistry with anti-S100A8 (clone 335580, R&D Systems). Immunofluorescence staining of 5 μm tumour sections was carried out as described previously40. To assess tumour vascularity, tumour sections were stained with CD31 antibody, followed by DAPI staining of nuclei. Images of stained sections were analysed for number of S100A8+ cells, number of DAPI+ nuclei, and area covered by CD31 with the help of ImageJ and Photoshop. The images shown in Fig. 1a and Supplementary Fig. 6 are representative of at least five randomly picked fields from multiple tumours.

Lung metastasis was quantified by bioluminescence imaging (luciferase–labelled tumour cells) or enumeration of visible lesions: on euthanization, animals were perfused with PBS, and lungs were excised and immediately subjected to bioluminescence measurement with IVIS Lumina II equipment. Then lungs were fixed in 4% PFA and visible lesions were counted with the aid of a magnifying stereoscope. Lung metastasis nodules were counted by two people who were blinded with regard to a sample’s experimental-group origin. Severity of metastasis was scored on the basis of these metastasis nodule counts. Paraffin-embedded lung samples were also submitted to haematoxylin and eosin (H&E) staining to reveal the size of lung metastases. For quantification, 10 stacked H&E-stained sections per lung, spaced 200 μm to cover a representative portion of the whole lung, were evaluated. Sections were scanned and total lung area and metastasis-covered area were measured using Photoshop. Figures 1k and 3h show the average level of lung metastasis burdens in their corresponding group (group sizes indicated in the corresponding figure legends).

Blood was drawn and collected in EDTA-coated tubes. To separate plasma from blood cells, a 15 mm centrifugation at 1,500g, 4 °C, was performed. Plasma was stored at −80 °C before analysis by Bioplex (23-cytokines-kit, Biorad) or ELISA (mouse G-CSF DuoSet, R&D Systems). Blood cells were subjected to red blood cell lysis, followed by FACS staining or other analyses.

Flow cytometry of blood and tumour samples. To characterize and quantify MDScs by FACS, cells were incubated with FcR blocker (eBioscience), and then stained with Ly6G–PE–CF594 (BD Biosciences), Ly6C–PerCP/Cy5.5 (BD Biosciences), CD11b–APC (eBioscience), and p4HA1 (BD Biosciences). MDScs were identified as Gr1+CD11b+ cells, granulocytic MDCs as Ly6G+Ly6C+CD11b+, monocylic MDCs as Ly6C+Ly6G–CD11b+. To determine absolute cell numbers counting beads (BD Biosciences) were added before FACS acquisition. T cells were analysed with an antibody combination of CD3–PerCPCy5.5, CD4–APC, CD8–FITC (all eBioscience) and PD1–PE–CF594 (BD Biosciences). The same antibody combination was also used to stain T cells. Tumour-infiltrating MDScs and T cells were quantified by enzymatic digestion of the dissected tumours (mouse tumour dissociation kit, Millenyi Biotech), followed by FACS staining with the above antibody combinations. Staining for the common leukocyte marker CD45 was also included to facilitate the distinction of leucocytes from tumour cells and other stromal cells.

Reverse-phase protein array. RPPA arrays were carried out as described previously41 with minor modifications. Protein lysates were prepared from tissue with Tissue Protein Extraction Reagent (TPER; Pierce) supplemented with 450 mM NaCl and a cocktail of protease and phosphatase inhibitors (Roche Life Science). Protein lysates at 0.5 mg ml−1 of total protein were denatured in SDS sample buffer (Life Technologies) containing 2.5% 2-mercaptoethanol at 100 °C for 8 min. The Aushon 2470 Arrayer (Aushon Biosystems) with a 40-pin (185 mm) configuration was used to spot lysates onto nitrocellulose-coated slides (Grase Bio-labs) using an array format of 96 (experimental and controls) lysates per slide with each sample spotted as technical triplicates (2,880 spots per slide). Slides were prepared for antibody labelling by blocking for 1 h with 1-Block reagent (Applied Biosystems) followed by 15 min incubation with Re-Blot reagent (Dako). Antibody labelling was performed at room temperature with an automated slide stainer Autolink 48 (Dako) using specific primary antibodies and appropriate biotinylated secondary antibody (Vector). An amplified fluorescent detection signal was achieved with Vectastain–ABC kit (Vector Laboratories) and Biotin Amp Reagent diluted at 1:250 (Perkin Elmer, NEL749B001KT) and a 1:50 dilution.
of LI-COR IRDye 680 Streptavidin (Odyssey). The total protein content of each spotted lysate was assessed by fluorescent staining with Sypro Ruby Protein Blot Stain for selected subset of slides (Molecular Probes). Fluorescent-labelled slides were scanned on a GenePix AL4200 scanner, and the images were analysed with GenePix Pro 7.0 (Molecular Devices). For normalization, raw image intensity of each spot is subtracted by that of negative control and then divided by total protein values. Tumours with different genetic backgrounds are analysed separately.

Western blotting. Tumour lysates described in the RPPA section above were also subjected to gel electrophoresis using 25 μg protein per lane of pre-cast 4–12% gradient minigels (Novex, Life Technologies), blocked with 5% dry milk in TBST for 1 h, shaking with supplier-recommended concentration of primary antibodies (all from Cell Signaling Technology) at room temperature for 4 h or at 4 °C overnight, and developing with secondary antibodies that were labelled for detection with the Odyssey system. Labelled molecular weight marker was also loaded on the gels. Protein bands were quantified using Odyssey features with scan times set to exclude overexposure. For representative images shown in Fig. 2b and Supplementary Fig. 2, at least three independent experiments were performed with similar images obtained.

In vitro T-cell activation assay. T cells of naive BALB/C mice were enriched from spleen by negative selection on magnetic beads (removal of TER119+, B220+ and CD11b+ cells) and labelled with CFSE (1 μM, Molecular Probes). T cells were cultured alone or admixed with MDSCs (at 1:0.33, 1:1 and 1:3 ratios) in 96-well plates. T-cell activation was with anti-CD3 (coating of wells at 5 μg ml⁻¹ overnight, 4 °C) and IL-2 (huIL-2, 2.5 ng ml⁻¹). At 5 days of culture, cells were collected and CFSE dilution was determined by FACS. Samples were also stained for Ly6G, to be able to exclude non-T-cells (MDSCs) from the analysis.

Cell culture supernatants were also collected and analysed by Bioplex assay of IFN-γ.

In vitro drug treatment. 4T1 cells were purchased from the Barbara Ann Karmanos Cancer Center. 220BL cells were derived from P53N-C tumours by continuous culture for more than one year. They were shown to express high E-cadherin levels suggesting the maintenance of luminal subtype of breast cancer characteristics and were apparently free of stromal components (only p53N genotype detected) at the time of use. Routine mycoplasma inspection was performed bi-monthly to ensure negativity of infection. No STR profiling or other authentication procedures have recently been performed. For drug treatment experiments, cells were seeded at 100,000 cells per well in 12-well format, or 10,000 cells per well of 96-well format the day before the experiment. Drug treatments were done after one wash in FBS-free DMEM (high glucose) medium for 8 or 24 h as indicated in the figures. BGI398 (Novartis), Torin 1 and latapinith stocks were prepared in DMSO at [1:0,000 of final concentration in ethanol. Control cells received corresponding amounts of vehicle alone. Cell supernatants were collected and, after a brief spin, analysed on G-CSF-ELISA plates (mouse G-CSF DuoSet, R&D Systems). In cell survival assays, WST-1 reagent (Roche) was added at end of the drug treatment period following the supplier’s instructions and attenuation "Dₐₚ" was measured.

Tumour initiation assay. P53N-C tumours were extracted and dissociated into single-cell suspension. FACS was employed to remove lineage-positive cells and dead cells. The remaining cancer cells were serially diluted into 50,000, 10,000, 2,000 and 400 cells ml⁻¹. A 100 μl cell suspension was transplanted into cleared mammary fat pads of recipient mice.

Mammosphere assay. Tumour cells (20,000 cells per well) were cultured in a low-adhesion 24-well plate in DMEM/F12 medium with murine bFGF (20 μg ml⁻¹, Life Technologies), murine EGF (20 ng ml⁻¹, Life Technologies) and B27 supplement (GIBCO). For co-culture, 80,000 Gr1+ MDSCs were added. As indicated, in some instances MDSCs and tumour cells were co-cultured in Boyden chambers, putting MDSCs on insets with 0.4-μm- pore-size membranes. Co-culture periods were 4–12 h for RNA expression study, 24 h for FACS of cancer stem cell surface markers, and 6–12 d to determine mammosphere-forming units. As indicated in the figures, specific control groups received Gr1+ cells of tumour-free mice (NNs, normal neutrophils) instead of Gr1+ MDSCs. These control cells were isolated from bone marrow with the same magnetic sorting procedure used for MDSCs (biotinylated anti-mouse Gr1, BD Pharmingen, Biotin Selection Kit, Stem Cell Technologies). Mammospheres were enumerated by manual counting of low-magnification images of the cultures (GenCount). The images in Supplementary Fig. 7a are representative of over 20 images from different experiments. For flow cytometry, mammospheres were dissociated by trypsin−EDTA digestion. Total tumour cell counts were obtained by FACS of the GFP-tagged tumour cells.

FACS of G-CSF, phospho-S6K, breast cancer stem cell markers. Single-cell suspensions of tumour cells were incubated on ice with FcR blocker (Ebioscience) in staining buffer (1% FBS in PBS), followed by cell surface marker staining with the following antibodies: CD24−PE, EpCAM−APC, Scal−PE, CD29−PECy7, CD49F−eFluor430, c-kit−APC as indicated in the figures. When intracellular staining was carried out, cells were fixed and permeabilized using reagents of Ebioscience’s FOXP3 staining kit. Intracellular staining was performed in the presence of 2% normal mouse serum, 2% normal goat serum and 2% FBS to reduce nonspecific binding. Antibodies used were G-CSF-APC (Ebioscience) and rabbit anti-p56k (T389, Cell Signaling), rabbit anti-p56k (5225/236, Cell Signaling) or rabbit anti-p53a (5727, Cell Signaling) followed by goat anti-rabbit-eFluor488.

For analyses of CSF1 first tumour issues, we first performed tumour dissociation using gentle MACS dissociators and tumour dissociation kit manufactured by Miltenyi Biotec. The cell suspension was then subjected to the staining described above. Owing to technical variations, tumours collected together and analysed at the same time were considered as an experimental set, and the systematic differences between different sets were removed by normalization (setting the mean frequencies of ‘No treatment’ samples as one within each set).

Co-culture of human bone marrow cells with human breast cancer PDX cells. Cell suspensions of PDX tumour cells were prepared with Miltenyi Biotec tumour dissociation kit as described above. To obtain tumour-cell-conditioned media, 1 × 10⁷ tumour cells ml⁻¹ were cultured for 3 days under mammosphere conditions (3D condition, low-adhesion plate, mammosphere medium described above) or on regular culture plates under 2D condition. MAMEM—high glucose with 10% FBS. Cell supernatant was collected, cleared by centrifugation and stored at −20 °C until use.

Unprocessed human bone marrow was obtained from healthy volunteers (Pietoics, consent agreement with vendor, Lonza). Red blood cells were removed by lysis.

In treatments with tumour-conditioned media, 1 × 10⁴ bone marrow cells per well of a 24-well plate were cultured in IMDM medium (IMDM, 10% heat-inactivated FBS, 0.01 M HEPES, 55 μM 2-mercaptoethanol, 1% antibiotic/anti-mycotic). To keep culture conditions consistent between groups, controls received 7.5% of 3D plus 7.5% of 2D non-conditioned tumour cell culture medium and experimental groups received 7.5% of 3D tumour-cell-conditioned medium plus 7.5% of non-conditioned 2D medium, or 7.5% of tumour-cell-conditioned medium plus 7.5% of non-conditioned 3D medium, respectively. Similar conditions were used with mouse bone marrow cells and mouse breast cancer cell line 4T1 were carried out under the same conditions. G-CSF-neutralizing antibody was added as indicated in the figures, at 6 μg ml⁻¹ (approximately five times ED₅₀ based on information provided by the supplier). Recombinant human G-CSF (Novoprotein) was used at 40 ng ml⁻¹ (specific activity: 6 × 10³ IU mg⁻¹).

After 3-day culture, cells were analysed by FACS. Cells with the phenotype of CD11b+CD33+CD15+CD16+HLA-DR−/− (human) or CD11b+Ly6G−(mouse) and high granulocytic characteristics in cytofluorimetric analysis were enumerated and assigned as granulocytic myeloid-derived suppressor cells.

Human bone marrow cells were also directly co-cultured with PDX tumour cells in a 4:1 ratio under mammosphere conditions. At the end of the 8-day culture period, the CD24 and CD44 profile of tumour cells was determined by FACS. Tumours and bone marrow cells were discriminated on the basis of FSC and SSC properties and CD11b.

PDX cell co-culture with MDSCs. PDX MC1 cell suspensions were prepared as described above, cleared of mouse stromal cells using mouse cell removal kit (Miltenyi Biotec) and mixed with purified MDSCs (Gr1⁺) from blood of MC1-tumour-bearing mouse and cultured under mammosphere conditions. The human cancer stem cell phenotype CD24−CD44⁺ was determined by flow cytometry with anti-human CD24−PE, CD44−eFluor488, anti-p56k(S389) (Ebioscience) and anti-human CD44−PE (Biologend) and included a mouse MDSC marker staining to facilitate the exclusion of this population by gating during data analysis.

Notch pathway studies. The γ-secretase inhibitor semagacestat (Selleck Biochem) was dissolved in DMSO as 10 mM stock and used at 10 μM final concentration. Controls received the same amount of DMSO. For assays of Notch pathway activation, the Notch reporter plasmid pCBFREluc, a gift from N. Gaiano, Johns Hopkins University School of Medicine, USA (Addgene plasmid no. 26897), was used. The Notch reporter was co-transfected with a constitutive expression (EFα promoter-driven) Renilla luciferase plasmid. Firefly luciferase (Notch reporter) and Renilla luciferase bioluminescence activity were measured by an in vivo imaging system (IVIS) and determined consecutively by adding respective substrates (D-luciferin, Gold Biotechnology and RediJect Coelenterazine h, PerkinElmer, MA, USA). Notch pathway activation was assessed by measuring luciferase activity in the day before and after reporter analyses. Notch reporter signal was normalized to the signal from constitutive plasmid. Dominant-negative forms of RBPJ (DN-RBPJ) and MAML (DN-MAML)
were applied to inhibit the intracellular Notch pathway. This was done either by cotransfection in the Notch reporter assays or by lentiviral transduction followed by GFP-based flow cytometric sorting to remove non-transduced cell population.

Bioinformatics analysis. The TCGA data set was obtained from the TCGA Data Portal in December 2012. Data for different platforms were matched on the basis of patient identifiers. Patients with incomplete records of DNA-copy number, mRNA expression, or RPPA data were excluded. The EMC-MSK data set was derived as described in the previous study[21]. The METABRIC data set[2] was described elsewhere and obtained from Cancer Research UK, Cambridge Research Institute. The FGFR signature was derived from a previous study by comparing MMTV-WNT and MMTV-WNT-igf2R tumours with MMTV-WNT tumours[21]. The genes that are significantly altered by inducible activation of FGFR1 were determined using a cutoff of FC (fold change) >2 and P < 0.05 (by two-sided Student’s t-tests). The signature was then applied to the gene expression profiles of p53-null tumours. Specifically, a single-value score was computed using the formula: \( \sum G^+ \left| \sum G^- \right| \), where \( G^+ \) represents the expression values of upregulated genes and \( G^- \) represents the expression values of downregulated genes. The G-CSF signature was derived on the basis of a previous study using the same approach as for FGFR signature. In Fig. 3b, we used GSE19222 to analyse the effect of an FGFR inhibitor, TKI258, on the expression of multiple cytokines. In Supplementary Fig. 4e, we used GSE12777 to gauge the correlation between G-CSF-sig and TCR genes in cell lines.

Gene Set Enrichment Analysis (GSEA) software was downloaded from the website http://www.broadinstitute.org/gsea/index.jsp. Gene signatures reflecting the action of pp242, rapamycin and G-CSF were derived from previous studies[21]. A continuous score of G-CSF signature was computed using a similar approach as described in the previous paragraph for the FGFR signature. GSEA was applied to the TCGA data set using genes up- or downregulated by pp242 and rapamycin as gene sets[6,7], and G-CSF signature scores as the continuous phenotype indices. P values of the enrichment were computed using built-in algorithms of GSEA based on random simulation.

Kaplan–Meier curve generation and tests (Fig. 4d), multivariate fitting of Cox proportional hazard model (Supplementary Fig. 4f), gene expression comparison across different molecular subtypes (Fig. 4c), and other data mining procedures and statistical assessments were performed using R Statistical Software on the basis of log-rank tests.

RT-qPCR. RNA was collected and processed in TRIzol, reverse transcribed to cDNA with Applied Biosystems’ High Capacity Reverse Transcription Kit, and analysed by real-time qPCR on an ABI7500 Fast Real-time PCR machine using SsoFast EvaGreen QPCR reagent (Biorad). Results were normalized to beta-actin and/or GFP (tumour cell label) and relative values were calculated as \( 2^{- \Delta \Delta C_{T} \text{sample} - \Delta \Delta C_{T} \text{reference}} \). Gene-specific primers were used listed in Supplementary Table 3.

Statistics and reproducibility. All results are presented in the form of mean ± s.e.m. unless otherwise specified. Sample sizes for in vivo experiments are noted in the corresponding figures or figure legends. Individual animals and independently prepared or treated primary tissue samples (for example, PDXs and primary human bone marrows) are considered as biological replicates. Different wells in one experiment are considered as technical replicates. In each experiment the size is determined on the basis of our prior knowledge of the variability of experimental output. Specifically, in animal experiments, a sample size of 4–8 mice per group allows us to detect a 50% difference in tumour size with power of 60–85% and \( \alpha = 0.05 \). Animals that suffered non-cancer-related disease conditions (for example, pathogen infection) were diagnosed by the Center of Comparative Medicine at Baylor College of Medicine on the basis of routine inspection, and excluded from experiments as per the recommendation of veterinarians based on pre-established protocols. Otherwise, animals were included into analyses. For the experiments shown in Figs 2g,h, and 3g, a randomization process was performed by randomly assigning animals into two separate groups. After assignment, tumour sizes of the two resultant groups were compared to make sure there is no statistically significant difference (by two-sided Student’s t-test). No randomization was performed in other experiments. The researchers were not blinded to allocation during experiments and outcome assessment except for experiment in Fig. 3f, in which the results were assessed by a researcher blinded to group allocation. For in vitro experiments including FACS analyses, in most cases two to three independent experiments were performed each with three to six technical replicates. Specific information is included in the corresponding figure legends. No specific tests were conducted to test the assumption of normal distribution. The difference between means of different experimental groups was analysed using two-tailed unpaired Student's t-test unless otherwise noted in respective legends. F-tests were performed to compare variation within different groups, and t-tests were performed with or without the assumption of equal variation accordingly. In a few experiments (see Fig. 7e,f), we computed \( P \) values in the framework of two-way ANOVA to achieve more stable estimates of biological variations by collectively analysing multiple biological conditions or perturbations. Specifically, in Fig. 7e, we treated different genetic perturbations (control, DN-Rbpj, and DN-Maml) as one factor, and different co-culture conditions (control, +MDSCs, and +NNs) as the second factor. In Fig. 7f, we used different co-culture conditions (with or without MDSCs) as one factor, different PDX models (HCl11 and MCl, left panel) or different treatments (with or without Notch inhibitor, right panel) as the second factor. Two-way ANOVA tested the null hypotheses that there is no significant difference among different levels of the same factor and there is no interaction between the two factors. The analyses were performed using the built-in two-way ANOVA analysis tool in Excel. Detailed output including results of post hoc tests is provided in Supplementary Table 4.

Accession numbers and data sets. Referenced accessions: previously published microarray data that were reanalysed here are available from GEO under accession codes GSE7400[22], GSE19222[21] and GSE12777[21]. The EMC-MSK data set[25] is comprised of GSE2603, GSE5327, GSE2034 and GSE12276. The METABRIC data set[2] is deposited at the European Genome-Phenome Archive (http://www.ebi.ac.uk/ega), which is hosted by the European Bioinformatics Institute, under accession number EGA5000000000083.

60. Achanyra, S. et al. A CXCL1 paracrine network links cancer chemoresistance and metastasis. Cell 150, 165–178 (2012).
61. Chang, C. H. et al. Mammary stem cells and tumor-initiating cells are more resistant to apoptosis and exhibit increased DNA repair activity in response to DNA damage. Stem Cell Rep. 5, 378–391 (2015).
62. Zhang, X. H. et al. Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma. Cell 154, 1060–1073 (2013).
63. Hoefflich, K. P. et al. In vivo antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models. Clin. Cancer Res. 15, 4649–4664 (2009).
Erratum: Oncogenic mTOR signalling recruits myeloid-derived suppressor cells to promote tumour initiation

Thomas Welte, Ik Sun Kim, Lin Tian, Xia Gao, HaiWang, June Li, Xue B. Holdman, Jason I. Herschkowitz, Adam Pond, Guorui Xie, Sarah Kurley, Tuan Nguyen, Lan Liao, Lacey E. Dobrolecki, Lan Pang, Qianxing Mo, Dean P. Edwards, Shixia Huang, Li Xin, Jianming Xu, Yi Li, Michael T. Lewis, Tian Wang, Thomas F. Westbrook, Jeffrey M. Rosen and Xiang H.-F. Zhang

Nature Cell Biology 18, 632–644 (2016); published online 16 May 2016; corrected after print 20 May 2016

In the version of this Article originally published, in the fourth affiliation, ‘Los Angeles’ should have read ‘Louisiana.’ This has been corrected in all online versions of the Article.
Supplementary Figure 1 Inter-tumoral heterogeneity of MDSC accumulation in mammary tumor models. a. Quantification of S100A8+ cells identified by immunofluorescence staining. Six fields were randomly picked from each tumor, and three tumors were included. b. Relative expression of the S100A8 gene in the bulk of tumor and in CD11b+Gr1+ cells purified from the tumor by FACS. n=2 and 5 tumors for the bulk tumor and purified CD11b+Gr1+ cells, respectively. c. In vitro CD3- and IL-2 stimulated T-cell proliferation, measured by CFSE decrease assay, is inhibited by MDSCs. The percentage of proliferating T-cells in conditioned medium (bottom) under indicated conditions. CD11b+Ly6G+ cells and T cells were admixed at 3:1. Two experiments each with technical triplicates were performed with similar results; results of one representative experiment are shown.
**Supplementary Figure 2** Genetic knockdown of Raptor in 4T1 cells as gauged by Western blotting against the indicated proteins.
Supplementary Figure 3 G-CSF inhibition reduces primary tumor growth and lung metastasis, an effect that is partially overcome by exogenous MDSCs. a. P53N tumor bearing mice were treated with G-CSF neutralizing antibody. Relative G-CSF levels in blood after treatment were measured by ELISA. n=5 and 3 animals in IgG and anti-GCSF groups, respectively. Two experiments were performed with consistent results, one representative experiment is shown. b. 4T1 tumor cells were lentivirus-transduced with G-CSF shRNA (Control: GIPZ vector transduced). Efficiency in reducing G-CSF levels in vivo (left panel) and in vitro (right panel) was evaluated by G-CSF ELISA. Left: n=10 and 17 animals for Control and shGCSF groups, respectively. Right: cells cultured in 3 different wells were used as technical replicates. Two independent experiments were conducted with representative results shown. c. MDSC numbers in blood of P53N-C tumor bearing mice after G-CSF-neutralizing antibody regimen compared to IgG control antibody-treated tumor-bearing mice. n=3, 4, and 7 animals for the three groups, respectively. P value is determined by non-parametric Wilcoxon test. d. 4T1 cells transduced with vector control lentivirus (GIPZ, control) or with G-CSF shRNA expressing lentivirus (shGCSF) were orthotopically injected to mammary gland. MDSC numbers in blood were quantified. n=9 and 7 animals for the two groups, respectively. e. No direct role of G-CSF in 4T1 tumor cell survival. Cell viability was assessed by WST-1 assay in presence of G-CSF neutralizing antibody or G-CSF shRNA compared to controls “n.s.”: no significant difference. One experiment was performed in which cells of different groups were cultured in 5 wells each. Error bars indicate S.E.M., and P values are calculated by two-tailed Student’s t tests unless otherwise noted. Statistics source data of Supplementary Fig. 3b-d are provided in Supplementary Table 4.
**SUPPLEMENTARY INFORMATION**

**Supplementary Figure 4** A G-CSF responsive gene signature (GCSF-sig) links the mTOR activity to MDSC infiltration in human breast cancer. a. Heat maps show the expression of G-CSF signature as a single score (top), and TCR pathway components (bottom) in the EMC-MSK dataset. The red sticks above the heat maps indicate tumor samples whose gene expression level exceeds the mean of all tumors by more than 2 x S.D. n=615 patients. b-d. Three human datasets were analyzed for the relationship between expression of G-CSF signature and T-cell activation signature in tumor biopsies. n=615, 409, and 1992 patients for b, c, d, respectively. e. As a negative control, the analysis was applied to a panel of tumor cell lines (n=51, available from GSE12777), showing no correlation as expected. For b-e, P values were determined based on two-side Student’s t tests for Pearson correlation coefficients. f. Multivariate (MV) analysis of METABRIC and EMC-MSK datasets using the Cox Proportional-Hazards model to estimate the hazard ratios (HR) of G-CSF-sig. The P values were computed based on the Multivariate Cox Proportional-Hazards model. g. MDSC quantity in P53N-C tumor-bearing hosts that are either wild type (WT) Balb/c mice or athymic nude (Nu) mice. TF (-) controls are shown for comparison. “n.s.”: no significant difference. n=5 animals per group. Error bars indicate S.E.M., and P values are calculated by two-tailed Student’s t tests. h. Representative Western blotting of pS6K(T389) and quantitative PCR assay of G-CSF in PDX tumors. Dotted lines indicate the matching between the two types of data across different PDX lines. Error bars indicate S.E.M.
Supplementary Figure 5. Correlation between high G-CSF expression and TIC features. a. 4T1 control cells and 4T1 cells expressing G-CSF shRNA were subjected to intracellular G-CSF FACS staining procedure. Overlay-graph demonstrates changes in GCSF levels. The experiment was performed twice with similar results. Results of a representative experiment are shown. b. Staining specificity control for pS6K antibody: PS6K levels were measured with similar results. Results of a representative experiment are shown. 

Supplementary Table 4.

© 2016 Macmillan Publishers Limited. All rights reserved.
Supplementary Figure 6 The mTOR-G-CSF-MDSC axis does not affect tumor angiogenesis. Immunofluorescence staining of CD31 was performed to quantitate angiogenesis in 4T1 tumors with indicated treatments. The CD31+ area was assessed using ImageJ software and displayed in the right panel. n=8, 7, 6, and 5 fields randomly picked from 4 different tumors for the four groups, respectively. n.s.: no significance. Scale bar, 100μm.
Supplementary Figure 7 MDSCs promote tumor-initiating capacity. a. Counts of mammosphere numbers with or without co-cultures of MDSCs. In each group, cells were cultured in four wells. Results shown are from one representative experiment among four independent experiments. Consistent trends were observed in all experiments. Scale bar, 100μm. b. Fold changes induced in 4T1 cells by co-culture with MDSCs are shown for total tumor cell numbers (GFP marker), and sub-populations of CD24<sup>hi</sup>CD29<sup>hi</sup>, CD49f<sup>+</sup>CD29<sup>hi</sup> and Sca1<sup>+</sup>c-Kit<sup>+</sup>. n=3 independent experiments per group, each experiment with technical triplicates. Combined results of all three experiments are shown. c. Mammary gland tumors formed by 4T1 cells that had been transduced with vector control (GIPZ) or with G-CSF shRNA lentivirus, were evaluated for mammosphere formation. For each tumor, cells were cultured in four technical replicates. Results (mammosphere numbers per equal tumor cell input) of two individual GIPZ control tumors and four G-CSF shRNA tumors are shown. d-e. Experimental design as shown in Fig. 8a. P53N-C tumor bearing mice were transiently treated with rapamycin or rapamycin plus MDSCs or left untreated. Tumor cells were extracted and tested for tumor initiation potential upon injection to mammary fat pads of untreated mice. Tumor size was measured on day 17 post tumor cell transfer from indicated groups (1,000 cell-injection per mouse). n=5 animals per group. Error bars, S.D. Kaplan Meier curve of TF survival. (200 cell injection per mouse). n=5 animals per group. P values were determined by log-rank test. Number of mice with tumors at end of observation time and total numbers of mice are also indicated for each condition. Error bars indicate S.E.M., and P values are calculated by two-tailed Student's t tests unless otherwise noted. Statistical source data of Supplementary Fig. 7b is provided in Supplementary Table 4.
Supplementary Figure 8. Unprocessed scans of Western blots. Images are annotated with sample identity in each lane and location of molecular weight markers. MW=molecular weight marker lane.
Supplementary Figure 8 continued

**Unprocessed scans for Supplemental Figure 2**

**Unprocessed scans for Supplemental Figure 3G**

---

Supplementary Figure 8 continued
Supplementary Tables

Supplementary Table 1 Reverse phase protein array (RPPA) reveals mTOR pathway activity as common denominator of breast cancer models that induce high MDSC accumulation. Table of RPPA data obtained by analysis of indicated tumor protein lysates as described in methods. All samples were measured in technical triplicates. Number of tumors analyzed per model (biological replicates) is indicated in second row of table. P53N-A: N=5, P53N-B: N=3, P53N-C: N=4, MMTV-Wnt1: N=4, Wnt1-iFGFR: N=4, P53-PTEN DKO: N=8

Supplementary Table 2 Antibody use information. The table lists for each antibody in this study from left: Name/antigen recognized, species-specificity, attached fluorochrome or biotin, clone ID, company providing the antibody, catalogue number, dilution used in experiment and type of experiment performed.

Supplementary Table 3 Gene-specific primers used in real-time qPCR. The table lists gene name and corresponding forward and reverse primer sequence, grouped into human and mouse genes.

Supplementary Table 4 Statistics source data. All data are organized into different sheets tagged by the corresponding figure/panel numbers. The same group identifiers were used as in the figures.

Supplementary Reference
Grubb, R.L., et al. Pathway biomarker profiling of localized and metastatic human prostate cancer reveal metastatic and prognostic signatures. J Proteome Res, 2009. 8(6):3044-3054. PMCID: PMC2790378.