



# The pleiotropic effects of TNF $\alpha$ in breast cancer subtypes is regulated by TNFAIP3/A20

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## Abstract

TNF $\alpha$  is a pleiotropic cytokine which fuels tumor cell growth, invasion, and metastasis in some malignancies, while in others it induces cytotoxic cell death. However, the molecular mechanism by which TNF $\alpha$  exerts its diverse effects on breast cancer subtypes remains elusive. Using in vitro assays and mouse xenografts, we show here that TNF $\alpha$  contributes to the aggressive properties of triple negative breast cancer (TNBC) cell lines via upregulation of TNFAIP3(A20). In a striking contrast, TNF $\alpha$  induces a potent cytotoxic cell death in luminal (ER+) breast cancer cell lines which fail to upregulate A20 expression. Overexpression of A20 not only protects luminal breast cancer cell lines from TNF $\alpha$ -induced cell death via inducing HSP70-mediated anti-apoptotic pathway but also promotes a robust EMT/CSC phenotype by activating the pStat3-mediated inflammatory signaling. Furthermore, A20 overexpression in luminal breast cancer cells induces aggressive metastatic properties in mouse xenografts via generating a permissive inflammatory microenvironment constituted by granulocytic-MDSCs. Collectively, our results reveal a mechanism by which A20 mediates pleiotropic effects of TNF $\alpha$  playing role in aggressive behaviors of TNBC subtype while its deficiency results in TNF $\alpha$ -induced apoptotic cell death in luminal breast cancer subtype.

## Introduction

Despite recent advances and better diagnostics, metastatic breast cancer is still incurable and remains the leading cause

of cancer-related death. Unlike the luminal and HER+ breast cancer patients for whom molecularly targeted therapeutics, such as the endocrine therapy and anti-HER2 agents are available, respectively, patients with the basal-like triple negative breast cancer (basal/TNBC) subtype have limited treatment options and currently lack molecularly targeted therapeutics. Women with basal/TNBC subtype constitute 15–20% of breast cancer patients and are often diagnosed with aggressive/metastatic disease [1, 2]. The basal/TNBCs are characterized by a distinct epithelial to mesenchymal transition (EMT) phenotype and cancer stem cell (CSC) properties [3], which we and others have shown to be driven by an inflammatory feedback loop [4–8]. Consistent with these studies, we demonstrated that simultaneous knockdown of TP53 and PTEN transforms MCF10A cells resembling the molecular and functional features of TNBC subtype [9]. The transformed MCF10A cells (MCF10A-p53<sup>-</sup>PTEN<sup>-</sup>) and TNBCs display a rapid proteolytic degradation of SOCS3, which resulted in activation of inflammatory cytokines and induction of EMT and CSC phenotype. In line with our findings, a genome-wide siRNA screen revealed that basal/TNBC subtype is highly addicted to proteasomal degradation [10].

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The TNF $\alpha$ -induced protein 3 (TNFAIP3 also called A20), a ubiquitin-editing enzyme, is originally identified as a protein protecting cells from TNF-induced cytotoxicity [11] and thus well-known for restraining excessive inflammation via its deubiquitinase (DUB) activity [12, 13]. Multiple autoimmune diseases such as lupus erythematosus are associated with polymorphisms in the A20 locus [12]. In addition to its DUB domain, A20 also exhibits E3-ubiquitin ligase activity by C2-C2 zinc-finger (ZF) motifs at the N terminal [12, 14]. TNF $\alpha$  signals through two receptors, TNFR1 and TNFR2, and activates NF- $\kappa$ B pathway in response to inflammation [15]. A20-deficient mice demonstrate spontaneous inflammation and premature death due to multi-organ inflammation and cachexia stemming from its inability to terminate NF- $\kappa$ B activity [16]. A20 is also required for the termination of TNF-independent inflammatory signals, such as Toll-like receptor (TLR)-activated NF- $\kappa$ B activity in macrophages [13]. However, some recent studies implicated a paradoxical role for A20 outside the immune system [17–20]. These context-dependent diverse functions of A20 may be attributed to its dual DUB and E3-ubiquitinating ligase activities [14, 21]. In a striking contrast, A20 has been reported to promote liver regeneration by activating inflammatory IL6/Stat3 signaling pathway via targeting SOCS3 for proteolytic degradation [19]. Consistent with these findings, elevated A20 expression, a poor prognostic factor in human cholangiocarcinoma, inversely correlated with reduced SOCS3 expression and activation of inflammatory Stat3 pathway [18]. Relative A20 overexpression in glioblastoma stem cells (GSCs) compared to non-stem glioblastoma cells is shown to play a role in maintenance of self-renewing GSCs, as well as protection from TNF-induced apoptosis [20]. Furthermore, overexpression and prognostic utility of A20 in multiple solid tumors has also been reported [18, 20, 22]. In line with these findings, a recent study demonstrated that elevated A20 levels in basal breast cancer subtypes promote the metastatic properties of this subtype by inducing an epithelial mesenchymal transition (EMT) phenotype via multi-monoubiquitylation of Snail1 [17].

Our studies here reveal that TNF $\alpha$ -induced A20 expression in TNBCs protects cells from cytotoxic cell death via upregulation of HSP70-mediated anti-apoptotic pathway while inducing an EMT/CSC phenotype by further activating inflammatory pStat3 pathway. Whereas in ER+ luminal cell lines, TNF $\alpha$  induces cytotoxic cell death due to their failure to induce A20-mediated HSP70 upregulation. We therefore provide evidence that A20 plays a critical role in regulating the pleiotropic effects of TNF $\alpha$ -induced responses in breast cancer subtypes.

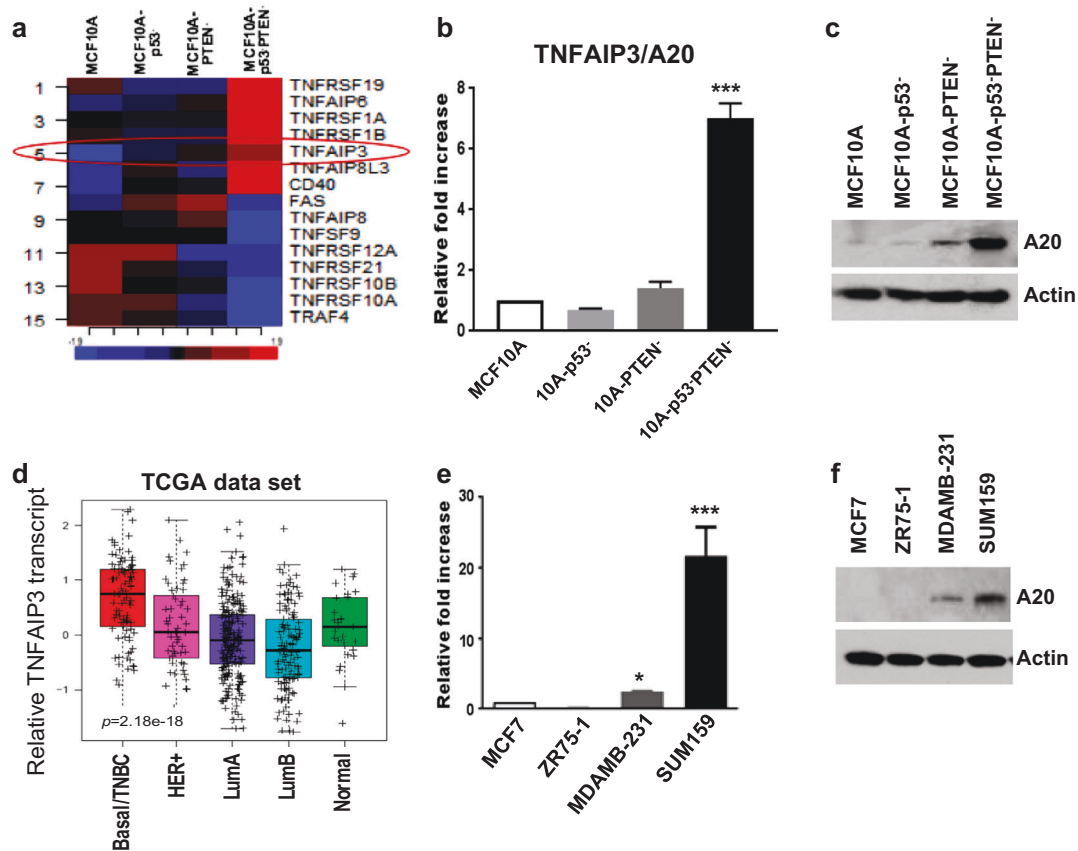
## Results

### A20 is highly upregulated in transformed TNBC model and patient TNBC subtype

We previously reported a transformed MCF10A model by simultaneous knockdown of p53 and PTEN (MCF10A-p53<sup>-</sup>PTEN<sup>-</sup>) which displays molecular properties of the basal/TNBC subtype [9]. One of the marked characteristics of MCF10A-p53<sup>-</sup>PTEN<sup>-</sup> cells compared to parental MCF10A or single gene deleted cells was the activation of inflammatory pathways and cytokines via proteolytic degradation of SOCS3. In an effort to identify E3-ubiquitin ligase that may be responsible for SOCS3 proteolytic degradation in MCF10A-p53<sup>-</sup>PTEN<sup>-</sup> cells, we re-examined our gene expression analyses, which revealed more than 500 differentially expressed genes between the parental MCF10A and MCF10A-p53<sup>-</sup>PTEN<sup>-</sup> cells. We found that TNF $\alpha$ -induced *A20 gene*, encoding E3-ubiquitin ligase is significantly upregulated in MCF10A-p53<sup>-</sup>PTEN<sup>-</sup> cells compared to parental and single gene deleted cells (Fig. 1a). Overexpression of TNFAIP3 in MCF10A-p53<sup>-</sup>PTEN<sup>-</sup> cells, compared with MCF10A, MCF10A-p53<sup>-</sup>, or MCF10A-PTEN<sup>-</sup> cells is validated by qPCR and Western blot analysis (Fig. 1b, c). Furthermore, A20 is significantly upregulated in human basal/TNBC subtype as shown by using TCGA data set (Fig. 1d). Elevated A20 transcripts and protein expression in basal/TNBC cell lines, MDA-MB231 and Sum159 cells compared to luminal MCF7 and ZR75.1 are confirmed by qPCR and western blotting (Fig. 1e, f).

### A20 protects luminal breast cancer cells from TNF $\alpha$ -induced cell death

TNF $\alpha$  is a pleiotropic cytokine which acts in a context-dependent manner [23]. However, the pleiotropic effects and mechanism of action of TNF $\alpha$  have not been well characterized in breast cancer subtypes. To test this, luminal and TNBC cell lines were treated with TNF $\alpha$  (50 ng/ml for 48 h) and examined for their apoptotic cell death by flow cytometry analyses based on Annexin V staining. Our data show that responses of luminal and basal/TNBC subtypes to TNF $\alpha$  stimulation are profoundly different. While TNF $\alpha$  induces a significantly high apoptotic cell death (over 70%) in luminal MCF7 and ZR75-1 cell lines, it shows no detectable apoptosis in basal/TNBC cell lines, MDA-MB231 and Sum159 (Fig. 2a and Supplementary Fig. 1a). We also confirmed that TNF $\alpha$  failed to induce cytotoxic cell death in our transformed MCF10A-p53<sup>-</sup>PTEN<sup>-</sup> cells when compared to parental MCF10A which showed more than 25% cell death (Supplementary Fig. 1b, c). In line with the preceding data, luminal breast cancer cell lines showed no



**Fig. 1** TNFAIP3/A20 is highly upregulated in transformed MCF10A-p53<sup>-</sup>PTEN<sup>-</sup> cells and patient basal/TNBC subtype. **a** TNF $\alpha$ -induced genes including TNFAIP3 (red circle) is highly expressed in MCF10A-p53<sup>-</sup>PTEN<sup>-</sup> cells. **b** Expression of TNFAIP3/A20 was verified by qPCR and **c** Western blot analyses. **d** TCGA data set

indicates the upregulation of A20 in Basal/TNBC breast cancer subtype. **e**, **f** Higher expression of A20 in TNBC cell lines, compared with luminal subtype was confirmed by Western blot and qPCR assays. Results are presented as mean  $\pm$  SD. \* $P < 0.05$ ; \*\*\* $P < 0.0001$

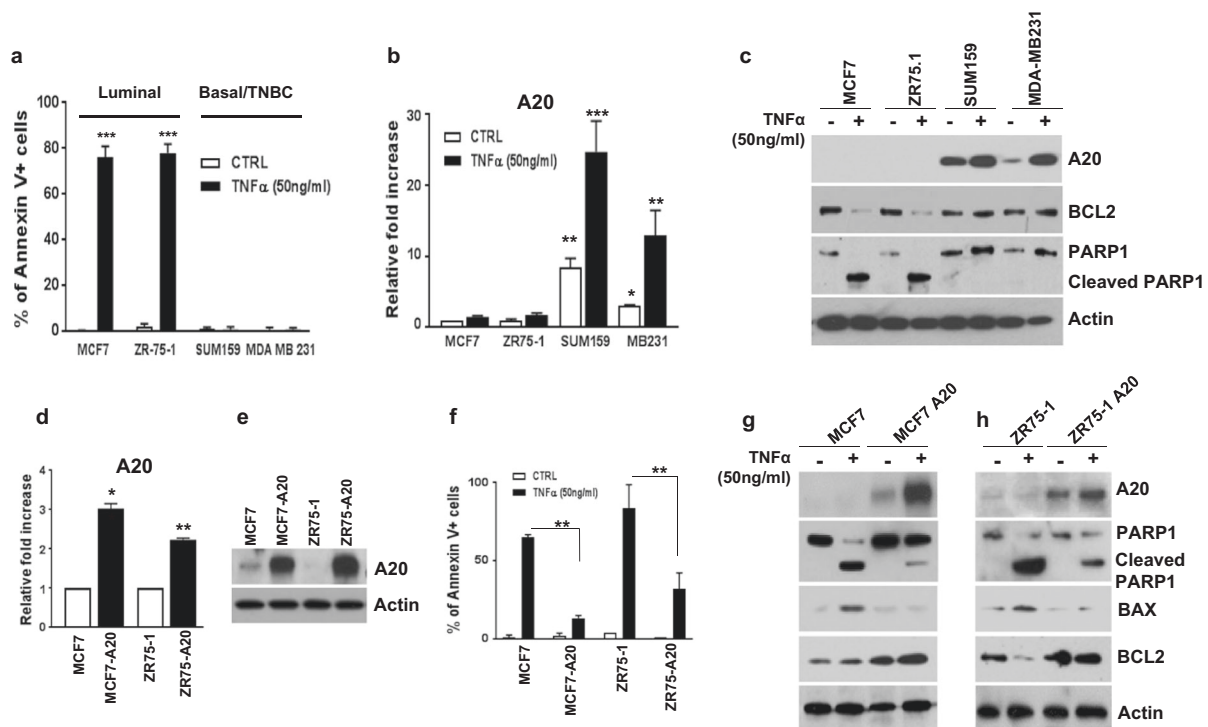
detectable A20 expression or induction in response to TNF $\alpha$  stimulation (Fig. 2b, c). In contrast, basal/TNBC cell lines showed a baseline A20 expression which was further enhanced in response to TNF $\alpha$  treatment (Fig. 2b, c). TNF $\alpha$ -induced cell death in luminal breast cancer cell lines correlated with an increased PARP1 cleavage and reduced anti-apoptotic protein BCL2, however, basal/TNBC cell lines showed a moderate increase in BCL2 level and there was no detectable PARP1 cleavage (Fig. 2c).

In order to investigate the molecular mechanisms of A20-mediated protection from TNF $\alpha$ -induced cell death, we generated A20 overexpressing stable clones of MCF7 and ZR75-1 cell lines. Overexpression of A20 in these cell lines is confirmed by qPCR analysis and western blotting (Fig. 2d, e). We then examined whether overexpression of A20 protects cells from TNF $\alpha$ -induced cytotoxicity. Indeed, A20 overexpression in both luminal MCF7 and ZR75-1 cell lines protected cells from TNF $\alpha$ -induced cytotoxicity while parental cell lines displayed a significant apoptotic cell death under identical conditions (Fig. 2f and Supplementary Fig. 2a). This A20-mediated protection from TNF $\alpha$ -induced cell

death in A20 overexpressing MCF7 and ZR75-1 cells is correlated with an increased level of A20 and anti-apoptotic BCL2 and reduced PARP1 cleavage and BAX (Fig. 2g, h).

### TNF $\alpha$ -induced post-translational upregulation of HSP70 is A20 dependent

Heat shock protein 70 (HSP70), one of the well-studied members of HSP family, is upregulated under conditions of cell stress and has been shown to protect cells from TNF $\alpha$ -induced cytotoxicity [24, 25]. We therefore investigated the possible involvement of HSP70 in A20-mediated protection of breast cancer cells from TNF $\alpha$ -induced cytotoxicity. The baseline HSP70 protein levels are comparable between A20 overexpressing MCF7 and ZR75-1 cell lines and their parental counterparts (Fig. 3a, b). However, TNF $\alpha$  significantly upregulated the HSP70 protein levels in MCF7-A20 and ZR75-1-A20 cell lines while it was substantially downregulated in their respective parental counterparts (Fig. 3a, b). Moreover, A20 protein levels are upregulated upon TNF $\alpha$  stimulation in A20 overexpressing cells suggesting a post-



**Fig. 2** A20 overexpression protects luminal breast cancer cells from TNF $\alpha$ -induced cell death. **a** Luminal and TNBC cell lines were treated with TNF $\alpha$  (50 ng/ml) for 48 h and examined for apoptotic cell death by flow cytometry analyses based on Annexin V staining. **b** Expression of A20 in TNF $\alpha$ -treated breast cancer cells was shown by qPCR assays. **c** TNF $\alpha$ -treated-induced expressions of A20, BCL2, and PARP cleavage were evaluated by western blot analyses. **d**, **e** Overexpression of A20 in MCF7 and ZR75-1 cells was confirmed by qPCR and

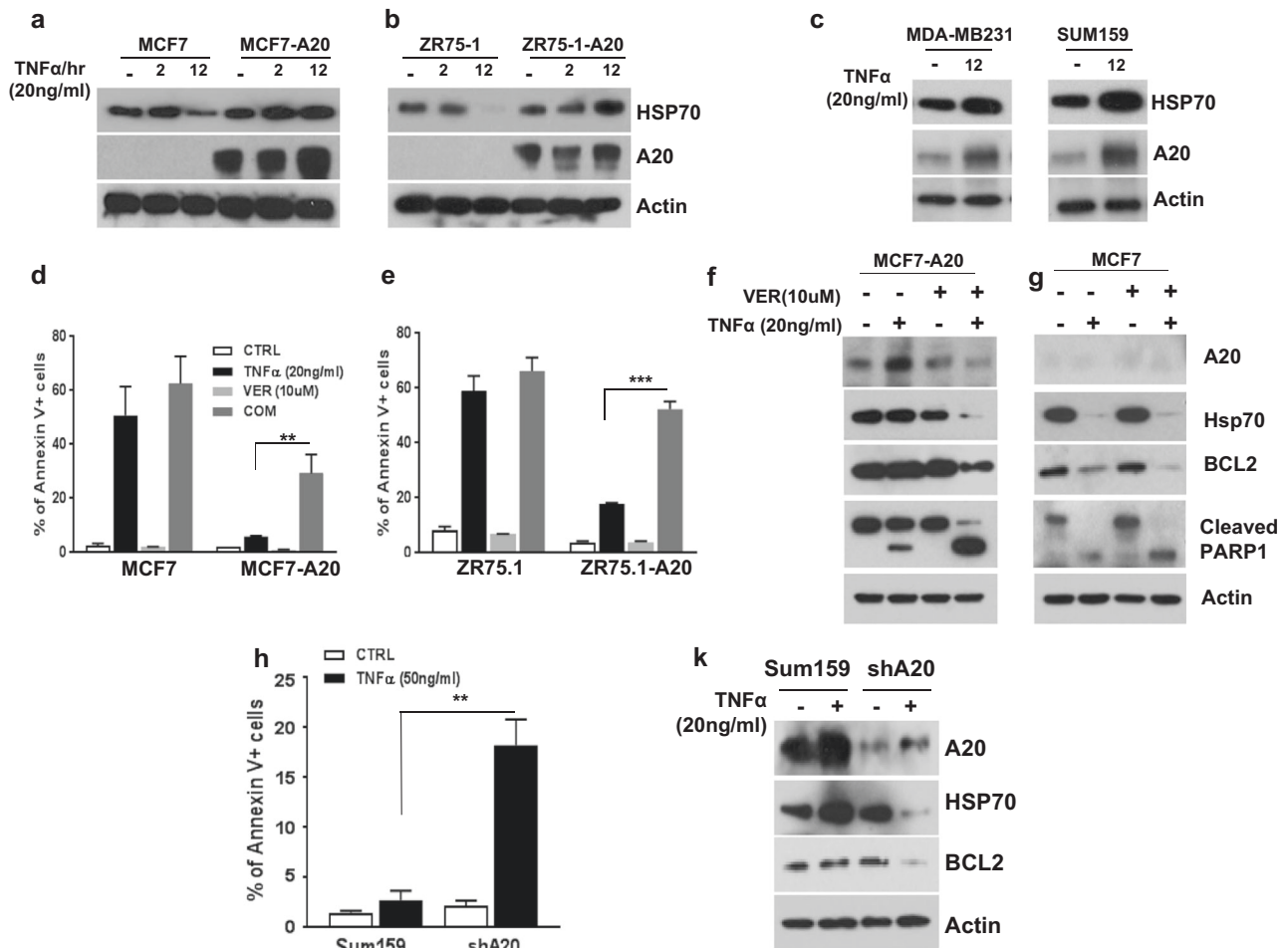
Western blotting. **f** Parental and A20 overexpressing MCF7 and ZR75-1 cells were treated with TNF $\alpha$  (50 ng/ml) for 48 h and analyzed cell death by Annexin V flow cytometry apoptotic cell staining. **g**, **h** Protein expression of A20, PARP cleavage, BAX, and BCL2 was shown in parental and A20 overexpressing cells with TNF $\alpha$  treatment. Results are presented as mean  $\pm$  SD. \* $P$  < 0.05, \*\* $P$  < 0.001, \*\*\* $P$  < 0.0001

translational protein stability (Fig. 3a, b). Consistent with the data, we further confirmed that TNF $\alpha$  induces the upregulations of A20 and HSP70 proteins in TNBC cell lines, MDA-MB231 and Sum159 (Fig. 3c). Altogether these data suggested that HSP70 may be involved in protection of cells from TNF $\alpha$ -induced cytotoxicity in an A20-dependent manner. To determine this, we stimulated cells with TNF $\alpha$  in the presence or absence of HSP70 inhibitor, VER155008. We found that the rate of apoptotic cell death did not significantly change when parental MCF7 and ZR75-1 cells were treated with combination of TNF $\alpha$  and VER155008 compared to the treatment with TNF $\alpha$  alone (Fig. 3d, e and Supplementary Fig. 2b, c). In contrast to parental cells, TNF $\alpha$ , when combined with VER155008, significantly increased the apoptotic cell death in A20 overexpressing cells which are resistant to TNF $\alpha$ -induced cytotoxicity (Fig. 3d, e and Supplementary Fig. 2b, c). We next determined that the combination of TNF $\alpha$  and VER155008 significantly downregulates the expressions of A20, HSP70 and anti-apoptotic BCL2 while increases the PARP1 cleavage in MCF7-A20 cells (Fig. 3f). Expectedly no significant changes were observed in parental MCF7 cells by inhibiting HSP70, because TNF $\alpha$  alone indeed downregulates

the expressions of HSP70 and anti-apoptotic BCL2 and induces PARP1 cleavage (Fig. 3g). In order to further verify the role of A20-mediated HSP70 in TNF $\alpha$ -induced cytotoxicity, we knocked down A20 in Sum159 cell line which represents TNBC subtype. The knockdown of A20 indeed renders Sum159 cells sensitive to TNF $\alpha$ -induced cytotoxicity (Fig. 3h) and that significantly correlates with reduced HSP70 and BCL2 protein levels (Fig. 3k).

### TNF $\alpha$ induces HSP70 degradation in luminal breast cancer subtype

Due to the fact that A20 is a ubiquitin-editing enzyme, next we investigated whether A20 regulates TNF $\alpha$ -induced HSP70 proteolytic degradation. We first determined that although basal/TNBCs cell lines showed significantly higher baseline HSP70 mRNA expression compared to luminal cell lines, they failed to increase the HSP70 transcription in response to TNF $\alpha$  stimulation (Fig. 4a, b). In contrast, luminal cell lines, MCF7 and ZR75-1 showed a significant upregulation of HSP70 mRNA upon TNF $\alpha$  stimulation (Fig. 4c). Interestingly, overexpression of A20 in

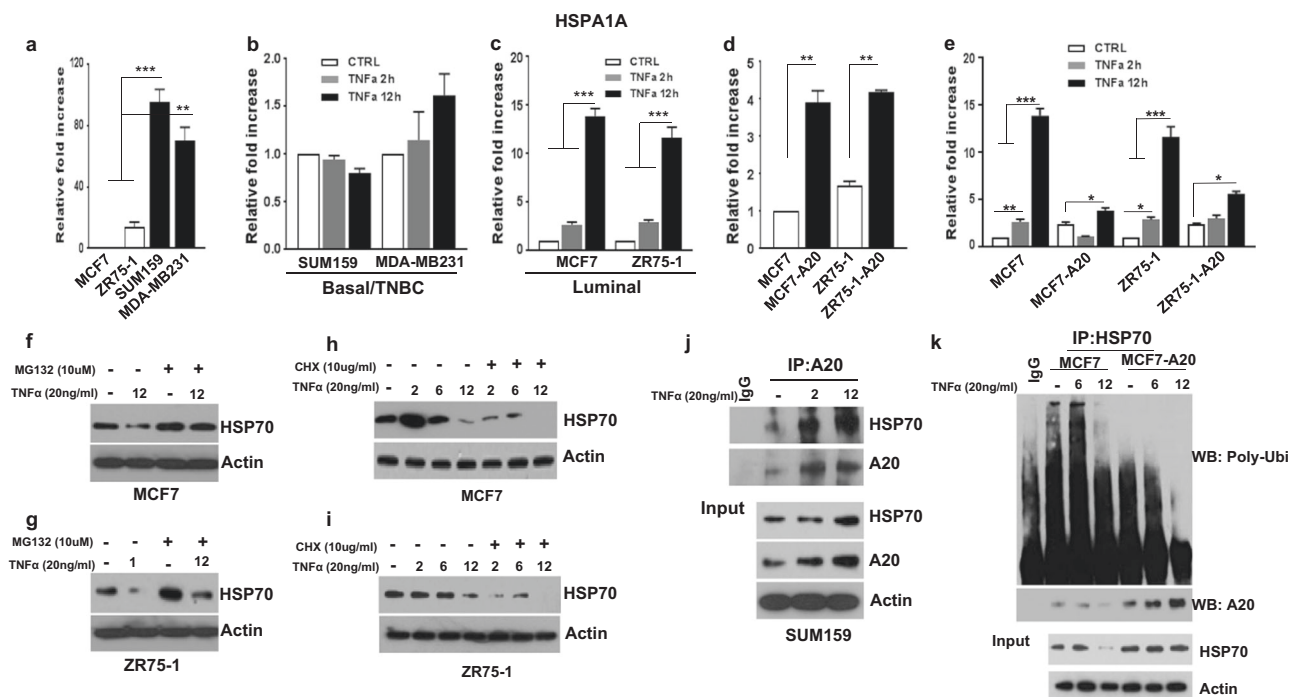


**Fig. 3** TNF $\alpha$ -induced post-translational upregulation of HSP70 is A20 dependent. **a, b** Parental and A20 overexpressing MCF7 and ZR75-1 cells were treated with TNF $\alpha$  (20 ng/ml) for 2 and 12 h and examined the protein expression of HSP70 and A20. **c** TNF $\alpha$  increased the expression of A20 and HSP70 proteins in MDA-MB231 and Sum159. **d, e** Parental and A20 overexpressing MCF7 and ZR75.1 cells were treated with TNF $\alpha$  (20 ng/ml) with or without VER155008 (10  $\mu$ M) for 24 h and analyzed cell death by Annexin V flow cytometry apoptotic

cell staining. **f, g** Protein expression of A20, HSP70, BCL2, and PARP cleavage was measured by Western blot assay. **h** Parental and A20-knockdown Sum159 cells were treated with TNF $\alpha$  (50 ng/ml) for 24 h and analyzed cell death by Annexin V flow cytometry apoptotic cell staining. **k** Protein expression of A20, HSP70 and BCL2 was measured by Western blot assay. Results are presented as mean  $\pm$  SD. \*\* $P < 0.001$ , \*\*\* $P < 0.0001$

luminal MCF7 and ZR75-1 cell lines increased the baseline HSP70 transcript levels albeit a reduced response to TNF $\alpha$  in A20 expressing cells compared to parental counterparts (Fig. 4d, e). These findings warranted further analyses of post-translational regulation of HSP70 in different breast cancer subtypes upon TNF $\alpha$  stimulation. It has been reported that HSP70 may be targeted for proteasomal degradation by E3 ligase [26, 27]. Consistent with these reports, TNF $\alpha$  induces the proteasomal degradation of HSP70 in luminal breast cancer cell lines, MCF7 and ZR75-1 as evidenced by the accumulation of HSP70 protein when cells were co-treated with MG132, an inhibitor of proteasome complex (Fig. 4f, g). This was further validated by the rapid HSP70 protein turnover when protein synthesis was inhibited by cycloheximide (CHX) in MCF7 and ZR75-1

cell lines upon stimulation with TNF $\alpha$  (Fig. 4h, i). These findings prompted us to examine the interaction between A20 and HSP70 in TNBC cell lines, which show upregulation of both proteins upon TNF $\alpha$  stimulation as shown in Fig. 3c. In line with our hypothesis, A20 binds to HSP70 in Sum159 cells as shown by immunoprecipitation (IP) and this interaction is enhanced when cells were treated with TNF $\alpha$  in a time-dependent manner (Fig. 4j). To determine poly-ubiquitination of HSP70, we performed an IP pull-down of HSP70 and immunoblotted with anti-poly-ubiquitin antibody. We indeed confirmed significantly higher levels of poly-ubiquitinated HSP70 in parental MCF7 cells upon TNF $\alpha$  stimulation, while this was reversed in A20 overexpressing MCF7 cells (Fig. 4k). We further corroborated these findings by overexpressing His-



**Fig. 4** TNF $\alpha$  induces HSP70 degradation in luminal breast cancer subtype. **a** Different mRNA expression of HSP70 in breast cancer cell lines was determined by qPCR. **b**, **c** Luminal MCF7 and ZR75-1 and basal/TNBC Sum159 and MDA-MB 231 cells were treated with TNF $\alpha$  (20 ng/ml) for 2 and 12 h and examined the HSP70 mRNA expression. **d**, **e** HSP70 mRNA levels were measured by qPCR in A20 overexpressing MCF7 and ZR75-1 cells with or without TNF $\alpha$  (20 ng/ml) treatment. **f**, **g** HSP70 expression in MCF7 and ZR75-1 cell was evaluated by Western blotting following treatment with TNF $\alpha$  (20 ng/ml) and MG132 (10 $\mu$ M). **h**, **i** MCF7 and ZR75-1 cells were treated

with TNF $\alpha$  (20 ng/ml) and CHX (10  $\mu$ g/ml) for 2, 6, and 12 h and examined the HSP70 protein expression. **j** Sum159 cells were treated with TNF $\alpha$  (20 ng/ml) for 2 and 12 h. Cell lysates were immunoprecipitated with anti-A20 antibody and Western blotting was performed with anti-HSP70 antibody. **k** MCF7 and MCF7-A20 cells were treated with TNF $\alpha$  (20 ng/ml) for 6 and 12 h and performed immunoprecipitation (IP) to pull-down HSP70 and immunoblotted with anti-poly-ubiquitin antibody. Results are presented as mean  $\pm$  SD. \* $P$  < 0.05; \*\* $P$  < 0.001; \*\*\* $P$  < 0.0001

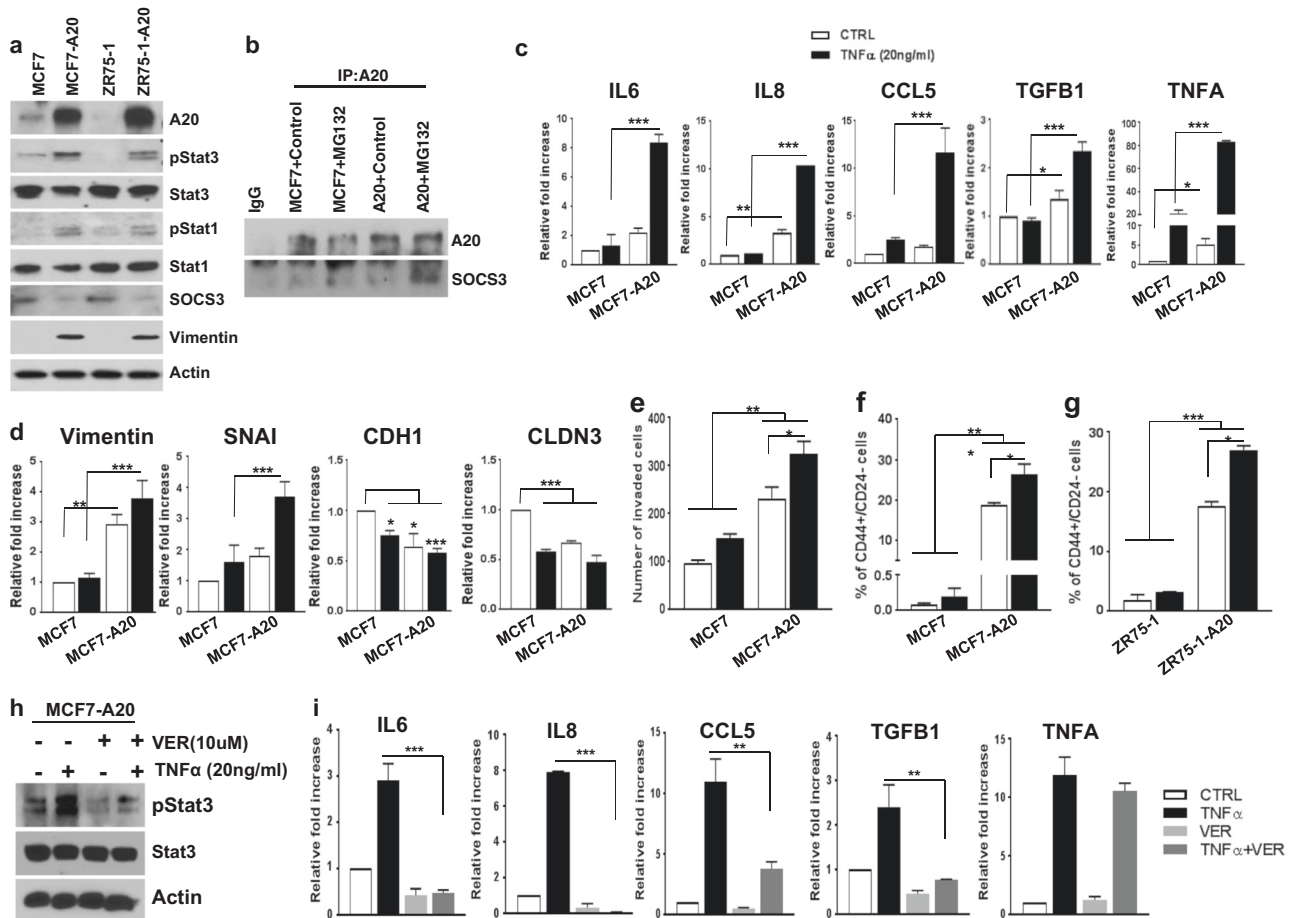
tag-Ubiquitin in parental MCF7 and MCF7-A20 cells. Notably, TNF $\alpha$  induced a significant poly-ubiquitination of HSP70 at 2 and 6 h time points in parental MCF7 cells compared to MCF7-A20 cells (Supplementary Fig. 3). Collectively our studies provide a compelling evidence that A20 protects HSP70 from TNF $\alpha$ -induced proteolytic degradation in luminal breast cancer subtype.

### TNF $\alpha$ -induced HSP70/A20/Stat3 pathway drives an EMT/CSC phenotype

The context-dependent paradoxical roles of A20-mediated activation of inflammatory IL6/pStat3 pathway via down-regulation of SOCS3 in liver regeneration and cancer have been previously reported [17–20]. We therefore examined the pStat1 and pStat3 pathways and expressions of cytokines in A20 overexpressing cells compared to their parental counterparts. MCF7-A20 and ZR75-1-A20 cells exhibited a higher Stat1 and Stat3 phosphorylation and downregulation of SOCS3 compared to their respective parental lines (Fig. 5a). To show whether A20 binds to

SOCS3 for possible proteolytic degradation, an IP was performed from lysates of MCF7 and MCF7-A20 cells treated with MG132, a proteasome inhibitor. Expectedly there was an increased interaction between A20 and SOCS3 protein in MCF7-A20 cells upon MG132 treatment (Fig. 5b). This suggested to us that A20 targets SOCS3 for proteolytic degradation. Consistent with the activation of inflammatory pStat3 pathway, A20 moderately increased the expressions of inflammatory cytokines, such as IL6, IL8, CCL5, TGF $\beta$ 1, and TNFA, which were further increased by TNF $\alpha$  stimulation compared to the parental cells (Fig. 5c and Supplementary Fig. 4a).

We and others previously demonstrated that activation of inflammatory cytokines induces an EMT phenotype and expands the CSC population [4, 6, 9, 28]. Therefore, we examined whether A20 overexpression in luminal breast cancer subtypes may contribute to induction of EMT/CSC phenotype. We evaluated expressions of EMT-related markers in parental and A20 overexpressing cells by qPCR and western blotting analyses. Both MCF7-A20 and ZR75-1-A20 cells showed an upregulation of EMT makers,



**Fig. 5** A20 overexpression induces the inflammatory Stat1/Stat3 pathway driving an EMT/CSC phenotype. **a** Expression of A20, pStat1, pStat3, SOCS3, and Vimentin was shown by Western blot in parental and A20 overexpressing MCF7 and ZR75-1 cells. **b** A20 pulls down SOCS3 protein in MCF7-A20 cells upon MG132 treatment compared to parental MCF7 cells. **c** mRNA expression of IL6, IL8, CCL5, TGFB1, and TNFA was shown by qPCR in parental and A20 overexpressing MCF7 cells after treating TNF $\alpha$ . **d** Expression of EMT and epithelial markers was analyzed by qPCR in parental and A20 overexpressing MCF7 cells with TNF $\alpha$  stimulation. **e** Transwell

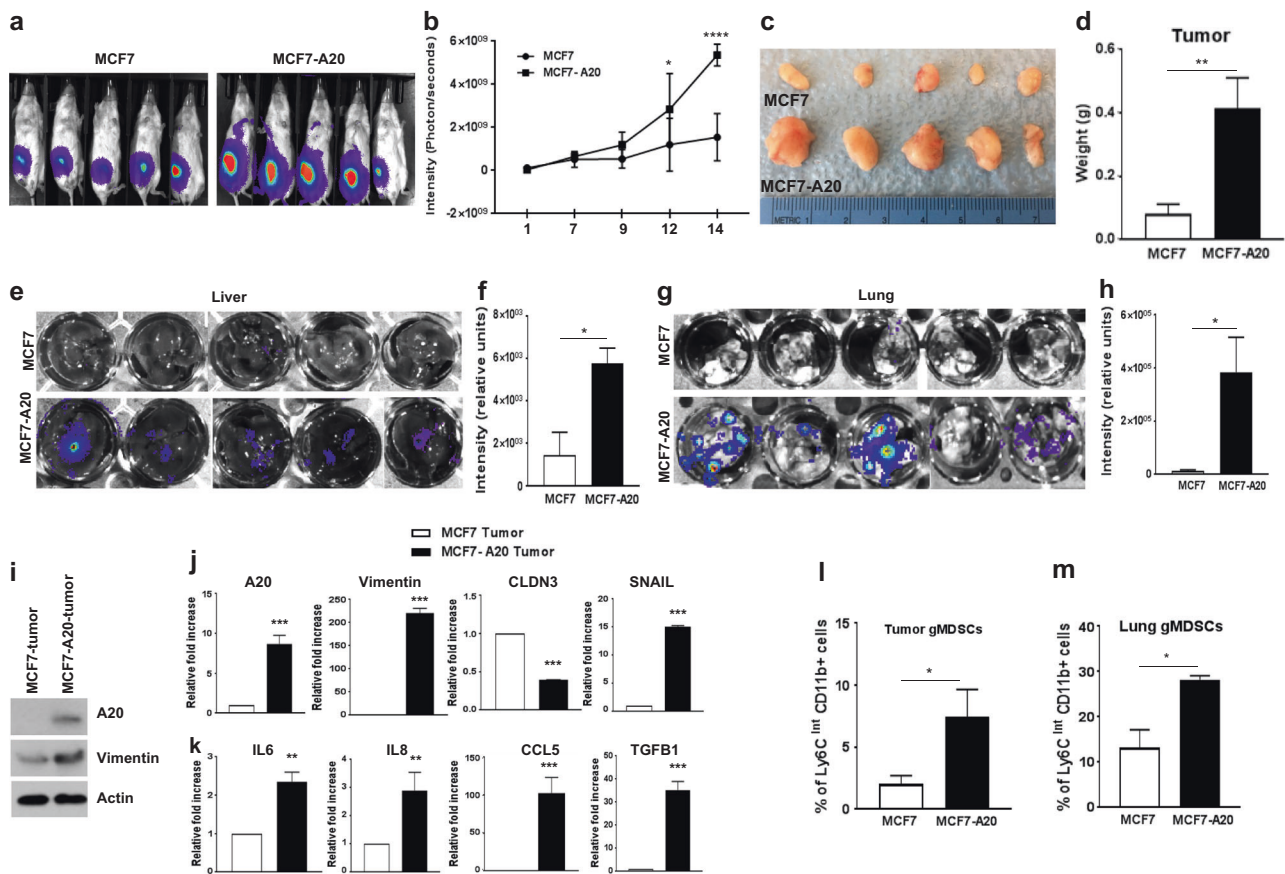
invasion assay showed an increased invasive ability of MCF7-A20 cells. **f, g** Flow cytometry analysis of CSC phenotype in A20 overexpressing MCF7 and ZR75-1 cells as assessed by CD44 and CD24 staining. **h** Expression of pStat3 and Stat3 was shown by Western blot in MCF7-A20 cells after treating TNF $\alpha$  and VER155008. **i** mRNA expression of IL6, IL8, CCL5, TGFB1, and TNFA was shown by qPCR in MCF7-A20 cells with TNF $\alpha$  and VER155008 treatment. Results are presented as mean  $\pm$  SD. \* $P$  < 0.05; \*\* $P$  < 0.001; \*\*\* $P$  < 0.0001

Vimentin and Snail and down regulation of epithelial markers, E-Cadherin and CLDN3 when compared to their parental counterparts (Fig. 5d and Supplementary Fig. 4b). Interestingly TNF $\alpha$  further enhanced the expression of EMT markers and downregulated the expression of epithelial markers in only A20 overexpressing cells (Fig. 5d and Supplementary Fig. 4b). Expectedly, A20 overexpression significantly increased the invasive potential of MCF7 cells compared to parental MCF7 and that TNF $\alpha$  stimulation further increased their invasive properties (Fig. 5e).

It is well established that the mesenchymal transition induces CSC phenotype in breast cancer cells [29]. To confirm the EMT-induced CSC expansion in our model, we examined the CSC content in parental and A20-expressing

cells by flow cytometry utilizing the CD44<sup>+</sup>CD24<sup>-</sup> phenotype. As expected, A20 expression significantly increased the CSC population, which was further expanded upon TNF $\alpha$  stimulation in both cell lines compared to the parental cells (Fig. 5f, g).

HSP70 has been shown to activate Stat3 phosphorylation and downstream signaling pathway [30, 31]. Therefore, we examined whether TNF $\alpha$ -induced HSP70 play any role in pStat3 phosphorylation in our model. On the basis of preceding experiments, inhibition of HSP70 by VER155008 significantly suppressed TNF $\alpha$ -induced pStat3 phosphorylation (Fig. 5h). In addition, the latter also resulted in downregulation of inflammatory cytokines, such as IL6, IL8, CCL5, and TGFb (Fig. 5i).



**Fig. 6** A20 overexpression promotes aggressive metastatic properties in mouse xenografts. **a, b** MCF7-Luc and MCF7-A20-Luc cells were orthotopically implanted into the mammary fat pads of NOD/SCID mice (five mice in each group). Enhanced primary tumor growth in MCF7-A20 mice was shown by bioluminescence imaging (BLI). **c, d** Primary tumors from MCF7 and MCF7-A20 tumor-bearing mice and their weights, which are presented in bar graphs are shown. **e–h**

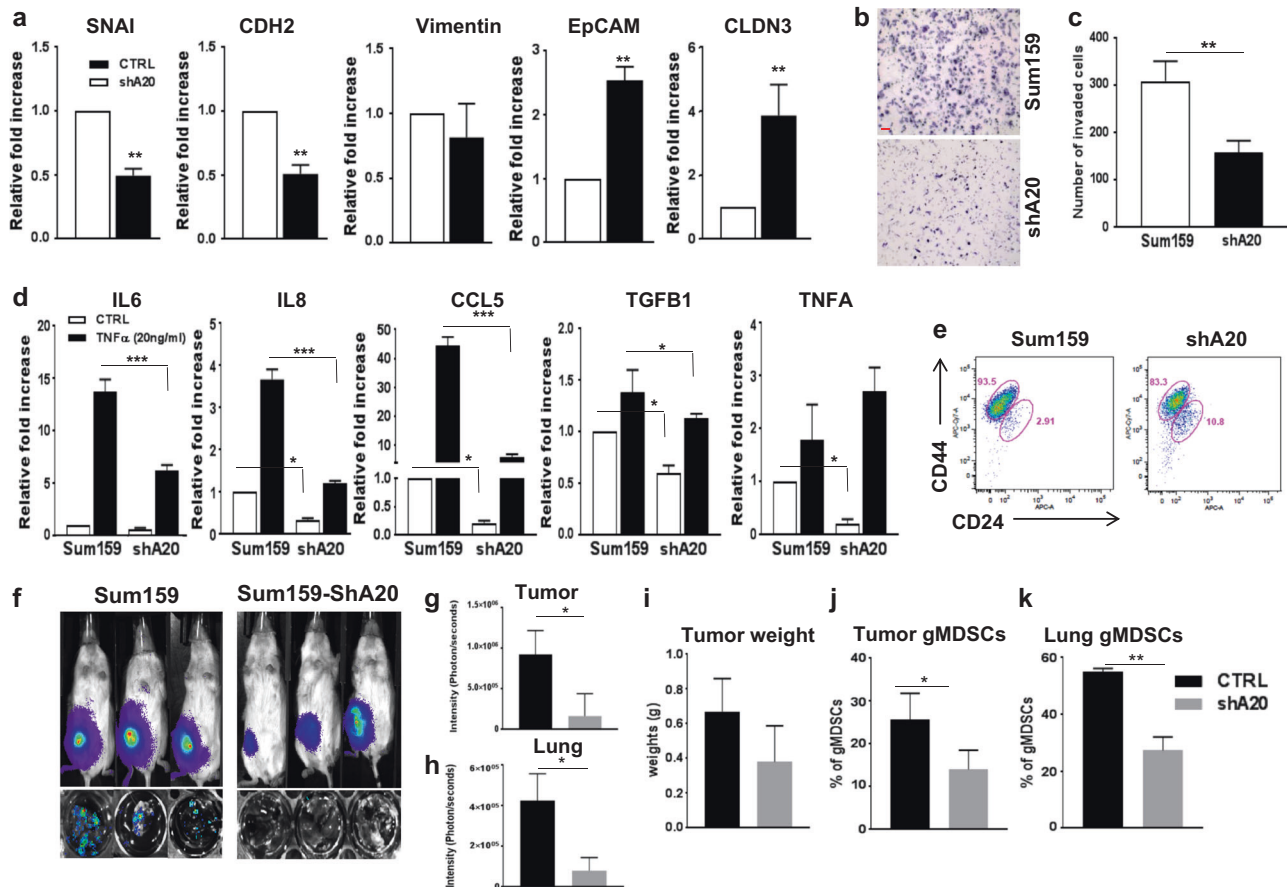
Enhanced metastasis in MCF7-A20 tumor-bearing mice were shown by ex vivo imaging of livers and lungs. **i–k** Expression of A20, vimentin, EMT markers, and cytokines in primary tumors was analyzed by Western blot and qPCR. **l, m** Primary tumor and lung infiltrating myeloid-derived suppressor cells were detected by flow cytometry analyses. Results are presented as mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.001$ ; \*\*\* $P < 0.0001$

### A20 promotes aggressive metastatic properties in mouse xenografts

Our in vitro findings suggested that A20 overexpression may promote aggressive properties of luminal breast cancer cells in mouse xenografts. To test their tumorigenic and metastatic potential, luciferase (Luc) expressing parental MCF7-Luc and stable clones of MCF7-A20-Luc cells were orthotopically implanted into the mammary fat pads of NOD/SCID mice. Tumor growth was monitored weekly in live animals by bioluminescence imaging (BLI). MCF7-A20-Luc xenografts exhibited a higher Luc signal intensity compared to the parental MCF7-Luc xenografts (Fig. 6a, b). An increased tumor size in MCF7-A20 tumor-bearing mice was also consistent with higher Luc signal intensity (Fig. 6c, d). Most importantly, all MCF7-A20 tumor-bearing mice exhibited a widespread metastasis in liver and lungs as demonstrated by ex vivo BLI of tissues (Fig. 6e–h). Molecular analyses confirmed overexpression of A20 and

upregulation of Vimentin in MCF7-A20 tumors compared to the parental MCF7 tumors (Fig. 6i). Elevated expressions of A20, EMT markers, and cytokines were further confirmed by qPCR analyses (Fig. 6j, k). We also determined that SOCS3 expression is suppressed in MCF7-A20 tumors compared to parental MCF7 tumors as assessed by immunohistochemistry staining (Supplementary Fig. 5a). Hematoxylin and eosin (H&E) staining of formalin-fixed paraffin embedded (FFPE) tissue sections exhibited metastatic lesions and immune infiltrates in lungs from MCF7-A20 tumor-bearing mice while no lesions were observed in lungs from MCF7 tumor-bearing mice (Supplementary Fig. 5b). We recently reported that infiltration of granulocytic myeloid-derived suppressor cells (gMDSC) in lungs promotes pulmonary metastasis [32]. We therefore examined whether pulmonary infiltrates in MCF7-A20 tumor-bearing mice are indeed gMDSCs. Flow cytometry analyses of dissociated lungs showed a higher pulmonary gMDSC infiltration in MCF7-A20 tumor-bearing mice compared to





**Fig. 7** A20 downregulation decreases EMT/CSC phenotypes in basal/TNBC Sum159 cells. **a** Expression of EMT genes and pro-inflammatory cytokines was analyzed by qPCR in parental and Sum159-shA20 cells with TNF $\alpha$  stimulation. **b, c** Transwell invasion assay showed a decreased invasive ability of Sum159-shA20 cells. Scale bar, 50  $\mu$ m. **d** A decreased expression of inflammatory cytokines, IL6, IL8, CCL5, TGFB1, TNFA in A20 downregulated Sum159 cells were shown by qPCR analyses. **e** Flow cytometry analysis of CSC phenotype in A20-depleted Sum159 cells as assessed by CD44 and

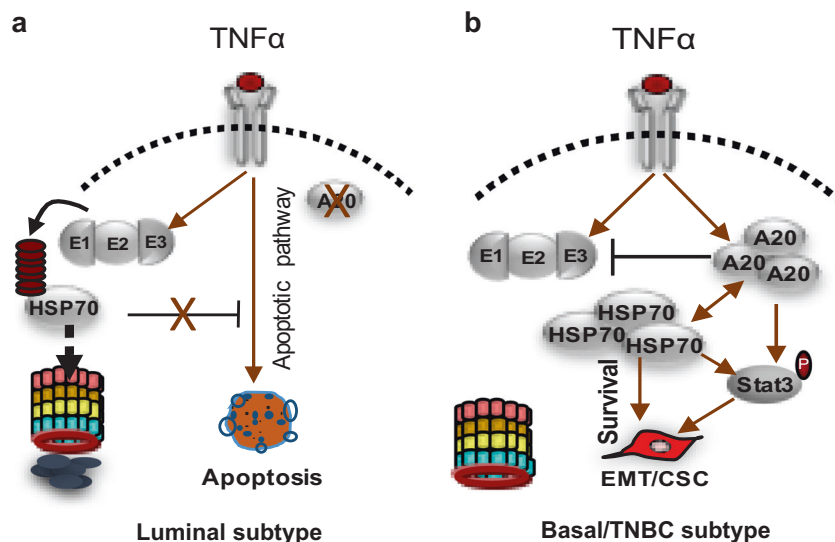
CD24 staining. **f–h** Sum159-Luc and Sum159-ShA20-Luc cells were orthotopically implanted into the mammary fat pads of NOD/SCID mice (three mice in each group). Suppressed primary tumor growth and lung metastasis in Sum159-ShA20 mice were shown by bioluminescence imaging (BLI). **i** Primary tumor weights which are presented in bar graphs are shown. **j, k** Primary tumor and lung infiltrating Myeloid-derived suppressor cells were detected by flow cytometry analyses. Results are presented as mean  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.001$ ; \*\*\* $P < 0.0001$

parental controls (Fig. 6l, m). We further confirmed and extended our findings using ZR75-1, another luminal breast cancer cell line. We surgically implanted Luc expressing parental ZR75-1 and stable ZR75-1-A20 cells into the mammary fat pads of NOD/SCID mice, monitored the tumor growth weekly by BLI and sacrificed the animals 10 weeks post-implantation. In accordance with the MCF7-A20 data, ZR75-1-A20 cells compared to the parental ZR75-1 generated larger primary tumors, as well as spontaneous pulmonary metastasis which may be due to increased gMDSC infiltrations in the lungs (Supplementary Fig. 6a–f).

To provide further direct evidence for the role of A20 in aggressive behaviors of breast cancers, we downregulated A20 in Sum159 cell line which represents TNBC subtype with a higher A20 expression. We generated stable clone by knocking down A20 via lentiviral short hairpin RNA

(Sum159-shA20 shown in Fig. 3k) which resulted in significant suppression of EMT markers, Snail, N-cad, and Vimentin, while increased the expressions of epithelial markers, EpCam and CLDN3 compared to parental Sum159 cells (Fig. 7a). The latter also correlated with reduced invasive properties of Sum159-shA20 compared to its parental counterpart (Fig. 7b, c). In addition, Sum159-shA20 cells not only showed reduced expressions of inflammatory cytokines, IL6, IL8, CCL5, TGFB1, and TNFA but also displayed decreased sensitivity to TNF $\alpha$  stimulation (Fig. 7d). Sum159 cells are well characterized TNBC subtype with a very high CSC compartment (over 90%) which may be attributed to the activation of multiple developmental pathways [6, 9, 33]. Down regulation of A20 in Sum159 cells was able to modestly reduce the CSC population (Fig. 7e). On the basis of our in vitro data, we next examined the tumorigenic and metastatic potential of

**Fig. 8** Schematic illustration of diverse TNF $\alpha$ -induced effects in luminal and basal/TNBC subtype. **a** TNF $\alpha$  induces an apoptotic signal via proteolytic degradation of HSP70 due to the lack of A20 in luminal breast cancer cell lines. **b** TNF $\alpha$  induces aggressive phenotype in basal/TNBC cells which are protected from apoptotic cell death via A20-mediated HSP70 upregulation



Sum159-shA20 cells. Orthotopically implanted Sum159-shA20 cells showed reduced primary tumor growth and pulmonary metastasis compared to the parental Sum159 cells (Fig. 7f–i). Furthermore, the failure of Sum159-shA20 tumors to show pulmonary metastasis may be due to failure to induce gMDSC infiltration in primary tumors and lungs (Fig. 7j, k). Together these studies with A20 knockdown in TNBC subtype further validated our data acquired using overexpression of A20 in luminal cells.

In Fig. 8, the summary of our data is illustrated to show that TNF $\alpha$  induces an apoptotic signal via proteolytic degradation of HSP70 due to the lack of A20 in luminal breast cancer cell lines. In contrast, A20 expressing basal/TNBC cells are not only protected from TNF $\alpha$ -induced apoptotic cell death but also acquire more aggressive phenotype by activating HSP70-mediated pStat3 inflammatory pathway.

## Discussion

We previously demonstrated that simultaneous knockdown of TP53 and PTEN transforms MCF10A cells displaying the molecular features of TNBC subtype, such as the induction of EMT/CSC phenotype [9]. The transformed MCF10A (MCF10A-p53<sup>-</sup>PTEN<sup>-</sup>) tumors in mouse xenografts exhibited aggressive properties, which we attributed to the activation of inflammatory cytokines via proteolytic degradation of SOCS3 [9]. The microarray analyses revealed several hundred genes that are differentially regulated in transformed MCF10A-p53<sup>-</sup>PTEN<sup>-</sup> cells compared to the parental MCF10A, single p53 or PTEN deleted clones. Among the highly upregulated genes, we identified TNFAIP3 (A20), a TNF $\alpha$ -induced gene which encodes a ubiquitin-editing enzyme [12]. The A20 gene was

of a particular interest because it was reported that it suppresses SOCS3 expression leading to activation of inflammatory pStat3 pathway [19]. We therefore demonstrated an inverse correlation between A20 and SOCS3 protein expressions in breast cancer cell lines representing luminal and basal/TNBC subtype. Moreover, overexpression of A20 in luminal cell lines suppressed the levels of SOCS3 protein and showed enhanced binding upon treatment with MG132, a proteasome inhibitor. Given the fact that basal/TNBCs characterized with activated inflammatory signature [6–8], A20 overexpression in this subtype may play critical role in their aggressive phenotype. Consistent with the notion, we showed that A20 transcript and protein levels are highly upregulated in MCF10A-p53<sup>-</sup>PTEN<sup>-</sup> cells and TNBC subtype compared to other subtypes as shown by using the TCGA data set. Furthermore, A20 has been recently shown to be overexpressed in basal-like breast cancers and play a critical role in aggressive metastatic properties of this subtype [17].

TNF $\alpha$ , a pleiotropic cytokine with an important role in inflammation and host defense, exerts diverse physiological functions depending on the cellular context [12, 13, 34, 35]. It induces an acute inflammatory responses via activating the inflammatory NF- $\kappa$ B pathway, which is suppressed by A20 to protect cells from cytotoxicity [11]. In contrast to its role in immune system, A20 has also been reported to play paradoxical roles in different tissues [17–20]. Supporting its paradoxical role, we demonstrated that A20 regulates the pleiotropic functions of TNF $\alpha$  in breast cancer subtypes. TNF $\alpha$  induces cytotoxic cell death in luminal breast cancer cell lines which lacks A20 expression, whereas it furthers the aggressive properties of basal/TNBC cell lines expressing elevated levels of A20. Furthermore, basal/TNBC cell lines further upregulate the A20 protein expression in response to TNF $\alpha$  stimulation by which they are protected

from cytotoxicity. These findings were further validated by overexpression of A20 in luminal breast cancer cell lines in which A20 not only protects them from TNF $\alpha$ -induced cytotoxic cell death but also induces aggressive properties in response to TNF $\alpha$  as shown by in vitro assays and mouse xenografts.

Although emerging data in cancer supports the notion that A20 may mediate TNF $\alpha$ -induced aggressive properties in some cancer types [17, 18, 20, 22], its central role in immune system is to protect immune cells from TNF $\alpha$ -induced cytotoxicity [11, 12]. The mechanism by which A20 protects immune cells from TNF-induced or TLR-induced apoptosis is by removing ubiquitin moieties from NF- $\kappa$ B or TRAF molecules [11, 13, 36]. However, other tissues such as lung, liver, kidney, and intestine may be protected from TNF-induced apoptosis by alternative mechanisms. It has been reported that HSP70 is upregulated in most tissues under conditions of cell stress and shown to protect cells from TNF $\alpha$ -induced cytotoxicity [37, 38]. A compelling evidence supporting the latter was provided by utilizing the *HSP70*-deficient mice which were no longer protected from the lethal effects of TNF-induced cytotoxicity [39]. We therefore hypothesized that TNF $\alpha$ -induced A20 may drive the inducible-HSP70 expression, which may protect basal/TNBCs from cytotoxicity. We show that both A20 and HSP70 protein levels were significantly upregulated in basal/TNBCs upon TNF $\alpha$  stimulation while their respective transcripts were not changed. In contrast however, luminal cells lacked A20 expression and failed to upregulate baseline HSP70 protein expression in response to TNF $\alpha$  stimulation albeit more than 10-fold increase in its transcript levels. These data were further corroborated by first using the A20 overexpressing luminal cell lines, which became resistant to TNF $\alpha$ -induced apoptosis and exhibited enhanced HSP70 protein expression. Moreover, inhibition of HSP70 when combined with TNF $\alpha$  in A20 overexpressing MCF7 cells resulted in apoptotic cell death. It was also confirmed by A20 knockdown in Sum159 cells which failed to induce HSP70 protein expression and were sensitized to TNF $\alpha$ -induced apoptosis. Furthermore, we determined that TNF $\alpha$ -induced upregulation of HSP70 is regulated via post-translational modification as there was no change at the level of *HSP70* transcript. Collectively our data suggest that TNF $\alpha$ -induced A20 binds to HSP70 and protects it from E3-ubiquitinating ligase-mediated proteasomal degradation. In turn, HSP70 may be involved in stability of A20 protein.

Although the HSF1-driven transcriptional activation of HSP70 is well documented in response to stress [40–42], its post-translational modification has not been well understood in response to TNF $\alpha$ . As previously reported [26, 27], we demonstrated in a series of experiments that TNF $\alpha$  induces HSP70 proteolytic degradation in luminal breast cancer cell

lines. However, A20 overexpression in luminal cell lines markedly reduced this process as evidenced by both a reduced poly-ubiquitinated HSP70 level, as well as an enhanced binding of A20 to HSP70 in response to TNF $\alpha$ . Furthermore, our data also suggest that A20 may be a client protein for HSP70 as evidenced by reduced levels of A20 protein when cells were stimulated with TNF $\alpha$  in the presence of HSP70 inhibitor. Our data is consistent with the role of HSP70 to promote the proteolytic removal of damaged or improperly folded proteins [25, 43].

Accumulating evidences suggest a paradoxical role for A20 in multiple solid tumors, as well as in liver regeneration [17–20, 22]. Despite the central role of suppressing inflammation in immune system, A20 has been shown to activate the inflammatory IL6/pStat3 pathway during liver regeneration via suppressing SOCS3 expression [19]. Furthermore, Lee et al. reported that A20 induces aggressive properties of basal-like breast cancer subtype by mono-ubiquitinating the Snail1 [17]. However, these studies negated the fact that A20 is a TNF-induced gene and failed to investigate changes in breast cancer subtypes in response to TNF $\alpha$ . We showed here that A20 expression was further upregulated in basal/TNBCs in response to TNF $\alpha$  promoting their aggressive properties as evidenced by marked increase in EMT/CSC phenotype. We and others provided evidence that inflammatory cytokines play a major role in induction of EMT/CSC phenotype [6–8, 29]. Consistent with these findings, A20 overexpressing cells further upregulated the expressions of inflammatory cytokines, such as IL6, IL8, CCL5, and TGF $\beta$  in response to TNF $\alpha$  stimulation. Upregulation of these inflammatory cytokines resulted in upregulation of EMT markers, as well as enhancing EMT/CSC phenotype. Interestingly A20 knockdown in basal/TNBC cell line reversed the expression of inflammatory cytokines, EMT markers, as well as reduced the EMT/CSC phenotype. Utilizing functional studies in mouse xenografts, we show that A20 overexpressing luminal breast cancer cell lines generated significantly larger tumors and spontaneous metastasis to liver and lung compared to their parental counterparts which grew smaller tumors and failed to show spontaneous metastasis.

In summary, our studies demonstrated how TNF $\alpha$  exerts its diverse effects on breast cancer subtypes. It furthers the aggressive properties of basal/TNBC subtype via A20 upregulation, whereas induces a cytotoxic cell death in luminal subtype which lacks the A20 expression. The mechanism by which A20 protects basal/TNBCs from cytotoxicity is by binding and enhancing HSP70 protein stability in response to TNF $\alpha$ . This results in induction of anti-apoptotic genes to overcome apoptotic cell death. In addition, A20 also promotes aggressive EMT/CSC phenotype by inducing the inflammatory Stat1 and Stat3 pathways

via both downregulating SOCS3 protein expression and upregulating HSP70 expression. Collectively our studies provide a compelling evidence that A20-mediated HSP70 may be an important molecular target for women with basal/TNBC subtype. Given the early clinical developments of HSP70 inhibitors, our studies provide a strong rationale for their therapeutic utility.

## Materials and methods

### Cell lines and reagents

MCF7, ZR75-1, MDA-MB-231, and Sum159 cell lines were purchased from American Type Culture Collection (ATCC). MCF7 and ZR75-1 cells were maintained in RPMI supplemented with 10% fetal bovine serum, and antibiotic/antimycotic 10,000 units/ml. MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% serum and antibiotic/antimycotic. Sum159 cells were maintained in Ham's F12 medium supplemented (with 5% fetal bovine serum, 5 mg/ml insulin, 1 mg/ml hydrocortisone, and antibiotic/antimycotic 10,000 U/ml). Recombinant human TNF $\alpha$  was purchased from Gemini Bioproducts. VER 155008, HSP70 inhibitor, was obtained from Sigma.

### Generation of A20 overexpression or knockdown cells

Lentiviral constructs were obtained from Applied Biological Materials and stable cell lines were obtained from manufacturer's protocol. Briefly, MCF7, ZR75-1, and Sum159 cells were transfected with 2  $\mu$ g of plasmid (pLenti-GIII-CMV-GFP-2A-Puro or piLenti-siRNA-GFP) with Transfection Reagent (Roche). Transduced cells were treated with puromycin and emerging colonies were selected and maintained in culture with puromycin.

### Annexin-V staining and flow cytometry

To detect apoptosis, cells were harvested in Annexin-V-binding buffer (Biolgend) or PBS with 2% FBS, stained with Annexin V-APC and 7-AAD and analyzed by a FACScanto flow cytometer (BD Biosciences). For CSC analysis, the cells were labeled with anti-CD44-APC-Cy7, anti-CD24-APC antibodies. All antibodies were purchased from Biologend.

### Western blotting and immunoprecipitation

Cells were lysed in RIPA buffer (Sigma). 50  $\mu$ g of each protein with Laemmli sample buffer were boiled for 5 min,

and subjected to SDS-PAGE. The proteins were transferred onto PVDF membrane (Bio Rad Laboratories) using semi-dry Trans-Blot (Bio Rad Laboratories). Blots were first incubated in TBS blocking buffer containing either 2% milk or 2% BSA (for phospho-specific antibodies) for 1 h at room temperature and then with the respective primary antibodies diluted in TBST (containing 0.1% Tween20 and 2% BSA) overnight at 4 °C. Subsequently, blots were washed and incubated with appropriate secondary antibodies (Santa Cruz) in TBST and detected using Super-Signal West Pico Chemiluminescent Substrate (Thermo).

For immunoprecipitation, cells were washed with cold PBS and lysed in lysis buffer (25 mM Tris pH 7.4, 120 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, and protein inhibitor cocktail). Cell lysates were incubated with appropriate antibodies at 4 °C for 12 h and with Protein A/G Magnetic Beads (Pierce) at room temperature for 1 h. Immuno-complexes were washed three-times with washing buffer, eluted by 2 $\times$  sample buffer and boiled for 5 min. Immunoblot analysis was subsequently performed. TNFAIP3/A20 antibody was purchased from Thermo Fisher. The antibodies to, BCL2, Cleaved PARP1, BAX, pStat3, pStat1 and SOCS3 were purchased from Cell Signaling Technology. HSP70 antibody was from Enzo Life Sciences.

### RNA extraction and real-time RT-PCR

Total RNA was extracted using RNeasy Mini kit (Qiagen) and 500 ng of RNA was used to make cDNA using iScript cDNA synthesis kit (Bio Rad). cDNA was analyzed in triplicate using real-time quantitative reverse transcription-PCR (qRT-PCR) assays (Bio-Rad). The information of the PCR primers and fluorogenic probes used are available on the Applied Biosystems website (GAPDH: Hs00266705, IL6: Hs00985641, IL8: Hs00174103, CCL5: Hs00174575, EpCAM: Hs00158980, Vimentin: Hs00185584, E-cadherin: Hs00170423, N-cadherin: Hs00983056, Claudin3: Hs00265816, Snail: Hs00195591, TGFb1: Hs00998133 and TNF: Hs01113624) KiCqStart SYBR Green pre-designed primers (Sigma) were used for the following genes: TNFAIP3/A20 (F: 5'-AGCCAATCATTGTCA TTTC-3', R: 5'-AGAACAATGGGGTATCTGTAG-3'), HSP70 (F: 5'-AATTCCTGTGTTTGAATG-3', R: 5'-AAAATGGCCTGAGTTAAGTG-3'). The relative fold change was measured by 2- $\Delta\Delta$ Ct formula compared to the control cells. Means and differences of the means with 95% confidence intervals were obtained using GraphPad Prism (GraphPad Software Inc.). Two-tailed Student's *t*-test was used for unpaired analysis comparing average expression between conditions. *P*-values < 0.05 were considered statistically significant.

## Transcriptome expression analysis

Total RNA was extracted with RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and reverse transcribed using the IVT Express kit (Affymetrix) and hybridized on Human Genome HG-U219 Strip Arrays (Affymetrix) comprised of more than 530,000 probes covering more than 36,000 transcripts and variants, which represent more than 20,000 genes mapped through UniGene or via RefSeq annotation.

## Invasion assays

Tumor cells ( $5 \times 10^4$ ) were seeded into the top chamber of transwell inserts coated with Matrigel (BD). The inserts were placed in a 24-well plate containing culture media of 5% FBS RPMI. Invaded cells were counted after 16–18 h. Experiments were done in triplicates.

## Mouse xenograft and tissue analyses

All mice procedures were conducted in accordance with the University Committee on the Use and Care of Animals. NOD/SCID female mice were purchased from Charles River at NCI. Parental and A20 overexpressing MCF7 and ZR75-1 cells (100,000 cells/fat pad) expressing the Luc gene were implanted into the fat pads of 5-week-old NOD/SCID mice. These mice were imaged utilizing the Caliper IVIS imaging systems. Primary tumors were fixed in pH7.4 buffered formalin and embedded in paraffin for histological or immunohistochemical examination. Lungs and livers were taken and processed for tissue histology to examine the metastatic lesions. Remaining fresh tumor tissues were dissociated and used for western blotting, PCR, and FACS analyses.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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