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Cancer-associated fibroblasts contribute to cisplatin resistance by modulating ANXA3 in lung cancer cells

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Cancer tissues consist of cancer cells, surrounding stromal cells and the extracellular matrix. Cancer-associated fibroblasts (CAF) are one of the key components of stromal cells. CAF have a great impact on the behavior of cancer cells, including proliferation, invasion, metastasis and chemoresistance in many ways. However, the underlying mechanism had not been fully elucidated. In this study, we investigated the role of CAF in cisplatin resistance of lung cancer cells. By using conditioned medium from CAF (CAF-CM), we found that CAF decreased the sensitivity of lung cancer cells to cisplatin. RNA sequencing results showed that CAF expressed a higher level of Annexin A3 (ANXA3) than normal fibroblasts (NF), and CAF-CM incubation increased the ANXA3 level in lung cancer cells. Overexpression of ANXA3 in lung cancer cells increased cisplatin resistance and activated c-jun N-terminal kinase (JNK), whereas knockdown of ANXA3 increased cisplatin sensitivity. Further study showed that CAF-CM enhanced cisplatin resistance by inhibiting cisplatin-induced apoptosis, determined by repression of caspase-3 and caspase-8, through activation of the ANXA3/JNK pathway. Conversely, suppression of JNK activation by specific inhibitor retarded the effect of CAF-CM and ANXA3 on cisplatin sensitivity. Taken together, our study demonstrated that CAF potentiated chemoresistance of lung cancer cells through a novel ANXA3/JNK pathway both in vitro and in vivo, suggesting ANXA3 could be a potential therapeutic target for the treatment of chemoresistant cancer.

KEYWORDS
ANXA3, cancer-associated fibroblast, cisplatin, JNK, lung cancer

1 | INTRODUCTION

Lung cancer is the most common malignant tumor worldwide, with the highest morbidity and mortality. There are approximately 1.8 million new cases and 1.6 million deaths annually, which account for 19% of all cancer deaths.[1] Non–small cell lung cancer (NSCLC) is the major pathological subtype of lung cancer, and 80%-85% of lung cancer cases are NSCLC. Nearly 70% of NSCLC patients present with advanced stage at the time of diagnosed,[2] leaving chemotherapy as the main treatment strategy. Currently, platinum-based chemotherapy the standard treatment for locally advanced NSCLC. However, clinical drug resistance remains a major issue, and the overall survival

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rate for NSCLC patients has not improved over the past three decades. Therefore, understanding the mechanism of drug resistance is crucial for better treatment of cancer patients.

Many factors contribute to the development of drug resistance in cancer. The genetic and epigenetic abnormity of cancer cells is the most commonly-studied mechanism underlying drug resistance.[3] Interestingly, recent studies have shown that not only the tumor cells themselves but also the tumor microenvironment (TME) may provide a protective niche for cancer cells from chemotherapy and lead to drug resistance.[4-6]

The TME consists of extracellular matrix (ECM), certain types of cells such as fibroblasts, neuroendocrine cells, adipose cells, immune-inflammatory cells, as well as the blood and lymphatic vascular networks.[7] CAF are among the major stromal cells in the TME. Although the origin of CAF is still under debate, accumulating evidence shows that several types of cells, including resident tissue fibroblasts, bone marrow-derived mesenchymal stem cells, hematopoietic stem cells, epithelial cells and endothelial cells, are possible predecessors of CAF.[8]

Cancer-associated fibroblasts can provide pro–survival signaling to the adjacent tumor cells by producing growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and stromal cell-derived factor-1 (SDF-1), in a paracrine manner.[9,10] However, the role of CAF in lung cancer chemoresistance has not been elucidated. In the present study, we have explored the effect of CAF on cisplatin resistance of lung cancers. We found that CAF decrease the chemosensitivity of lung cancer cells through inhibiting cisplatin-induced apoptosis by activating the ANXA3/JNK pathway.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

Cisplatin (CDDP) was purchased from Sigma-Aldrich (St Louis, MO, USA). JNK inhibitor SP600125 was purchased from Sigma-Aldrich and dissolved in DMSO. Antibodies used for western blotting were rabbit anti–ANXA3 (Saierbio, Tianjing, China), rabbit anti–phospho-JNK (Cell Signaling Technology), rabbit anti–survivin (Abcam, Cambridge, MA, USA), mouse anti–caspase 8 (Cell Signaling Technology), rabbit anti–caspase 3 (Cell Signaling Technology) and mouse anti–β-actin (Sigma-Aldrich). The secondary antibodies coupled to HRP were purchased from ZSGB-BIO (Beijing, China). Recombinant Human ANXA3 was purchased from R&D (Minneapolis, MN, USA). ANXA3 neutralizing antibody was purchased from Sigma-Aldrich.

2.2 | Cell culture

Human lung cancer cell lines A549, H661 and SK-MES-1 were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown and maintained in DMEM medium (GIBCO BRL, Grand Island, NY, USA) at 37°C, 5% CO_2. Medium was supplemented with 10% FBS.

2.3 | Isolation and culture of stromal fibroblasts

Cancer-associated fibroblasts were isolated from primary tumor tissue as previously described.[11] CAF were cultured in DMEM/F12 medium supplemented with 10% FBS at 37°C, 5% CO_2. For the preparation of CAF-conditioned medium (CAF-CM), CAF were cultured for 48 hours; the CAF-CM was collected and centrifuged for 10 minutes at 1500 g to remove cell debris. All in vitro experiments were performed in triplicate and CAF were at <10 passages.

The lung cancer tissues were obtained from patients at Tianjin Medical University General Hospital (TMUGH, Tianjin, China), who underwent surgery without chemotherapy treatment history. Informed consent was obtained from all patients for the collection and use of specimens, and the study was approved by the Institutional Review Board of TMUGH.

2.4 | Cell viability assay

Cell viability was assessed by using the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) following the manufacturer's instructions. Briefly, lung cancer cells were plated at a density of 8-10 × 10³ cells/well in a 96-well plate; they were then treated with 0-80 μmol/L CDDP for 48 hours. Cell viability was detected by CCK-8, and the median inhibitory concentration IC_{50} values were calculated using GraphPad Prism 5.0 software (La Jolla, CA, USA).

2.5 | Flow cytometric evaluation of apoptosis

Lung cancer cells were treated with CDDP for 24 hours. After the treatment, the apoptotic cells were determined using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA), following the manufacturer's instructions. Briefly, cells were washed with PBS and resuspended in binding buffer. Annexin V-FITC and PI were then added to the cells, before incubation for 15 minutes at room temperature in the dark. The apoptosis analysis was performed on a FACSAria flow cytometer (Becton Dickenson, San Jose, CA, USA).

2.6 | RNA interference and transfection

The siRNA duplexes were purchased from Genepharma (Shanghai, China). The sequences of siRNA duplex for ANXA3 were: sense: 5′-GG- ACAAGCAGGCAAAUGAATT-3′, anti–sense: 5′-UUCAUUU GCUUGUCCTT-3′. Lung cancer cells were plated into 6-well plate at a density of 2.5 × 10⁵ cells/well, transfected with siRNA duplexes with Lipofectamine 2000 (Invitrogen, California, USA), and incubated for 48 hours before further analysis.

We constructed the plasmid of pcDNA3.1(+)-ANXA3 ourselves. Lung cancer cells were plated into 6-well plate at a density of 2.5 × 10⁵ cells/well; 2 μg of pcDNA3.1(+)-ANXA3 was transfected into A549 and H661 cells with Lipofectamine 2000 and incubated for 48 hours before further analysis.

2.7 | Quantitative PCR

Total RNA was extracted from cells or tissues using Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed by using a TaKaRa Kit (Dalian, China) according to the manufacturer's instructions. The gene expressions were measured by quantitative PCR (qPCR) using Power SYBR Green Master Mix (ABI, Foster City, CA, USA) on an ABI Prism 7900HT Sequence Detector System (ABI). The primers for ANXA3 were: forward ACAGCGGCAGCTGATTGTTA; reverse TCACTAGGGCCACCATGAGA. PCR reactions were performed as previously described,[12] under the following conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 34 seconds. GAPDH was used as an internal control.

2.8 | Western blotting

Western blotting was performed as previously described.[13] Briefly, protein was extracted from cells using a RIPA lysis buffer containing protease inhibitor (Sigma-Aldrich). The proteins were separated by running a 12% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non–fat milk for 1.5 hours at room temperature. Then the membranes were probed with primary antibodies at 4°C overnight and further incubated with the HRP-conjugated secondary antibodies at 37°C for 1.5 hours. Finally, the protein bands were visualized using the ECL Western Blotting System following the manufacturer's instructions.

2.9 | RNA sequencing

Total RNA was extracted from CAF and NF *using the RNeasy Kit (Qiagen), following* the manufacturer's instructions. The RNA sequencing was conducted on a BGISEQ-500 by BGI (Shenzhen, China).

2.10 | In vivo tumor studies

BALB/c nude mice were obtained from the Cancer Institute of the Chinese Academy of Medical Science (Beijing, China). Xenograft experiments were conducted in accordance with the Tianjin Medical University Institutional Animal Care and Use Committee guidelines. Each treatment group consisted of 5 mice; 3×10^6 A549 cells or 3×10^6 A549 cells mixed with 9×10^6 CAF (ratio1:3) were resuspended in 100-μL PBS and injected subcutaneously into the mice. Beginning 1 week after injection, tumor masses were measured every week, and tumor volume was calculated as: volume $(mm^3) = d^2 \times D/2$, where the d and D were the shortest and the longest diameters. When the tumor volume reached approximately 50 mm^3, mice were treated with CDDP (3 mg/kg body weight), whereas the control group was treated with normal saline. After
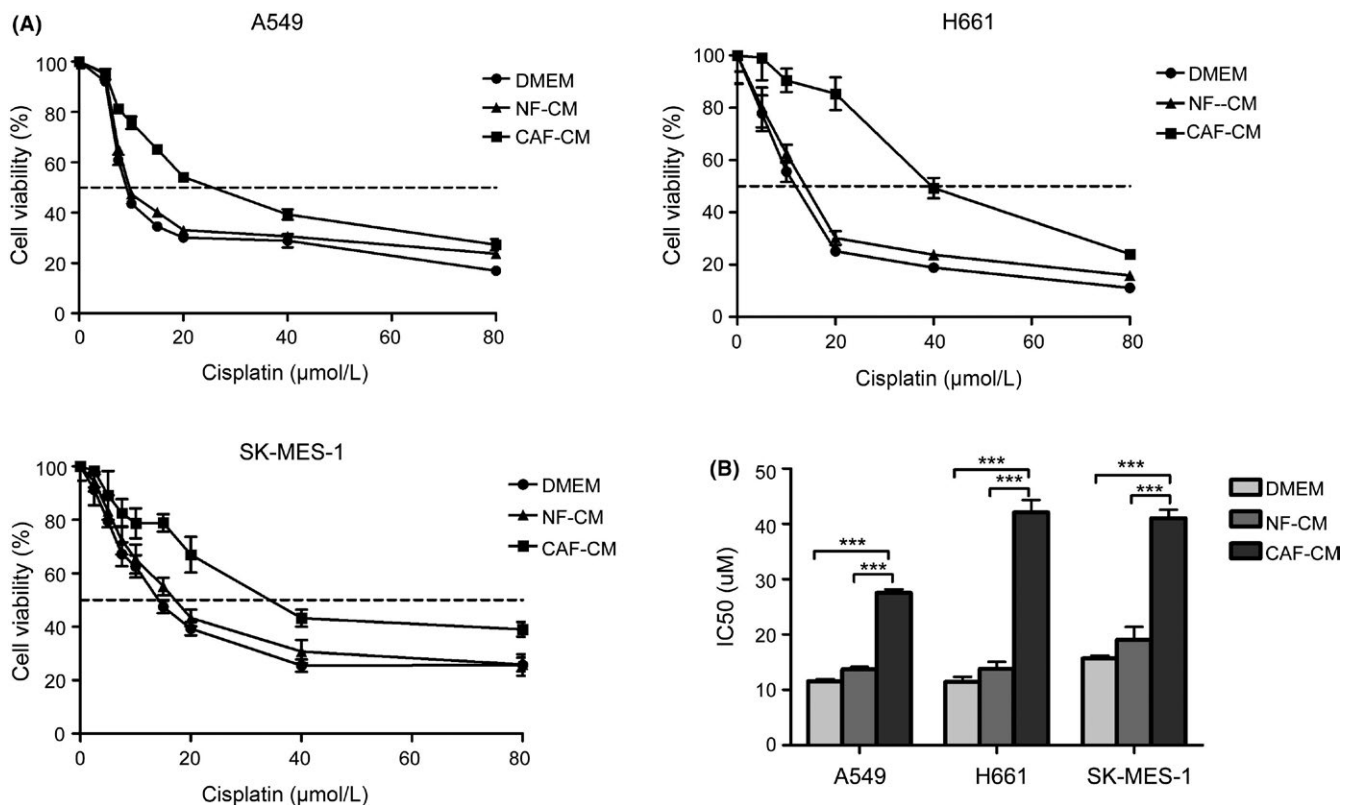

FIGURE 1 Cancer-associated fibroblasts (CAF) enhanced cisplatin resistance of lung cancer cells. Lung cancer cells were cultures in CAF-CM and treated with 0-80 μmol/L cisplatin for 48 h. Cell viability was detected by CCK-8 assay. Values represent the mean ± SD from 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001
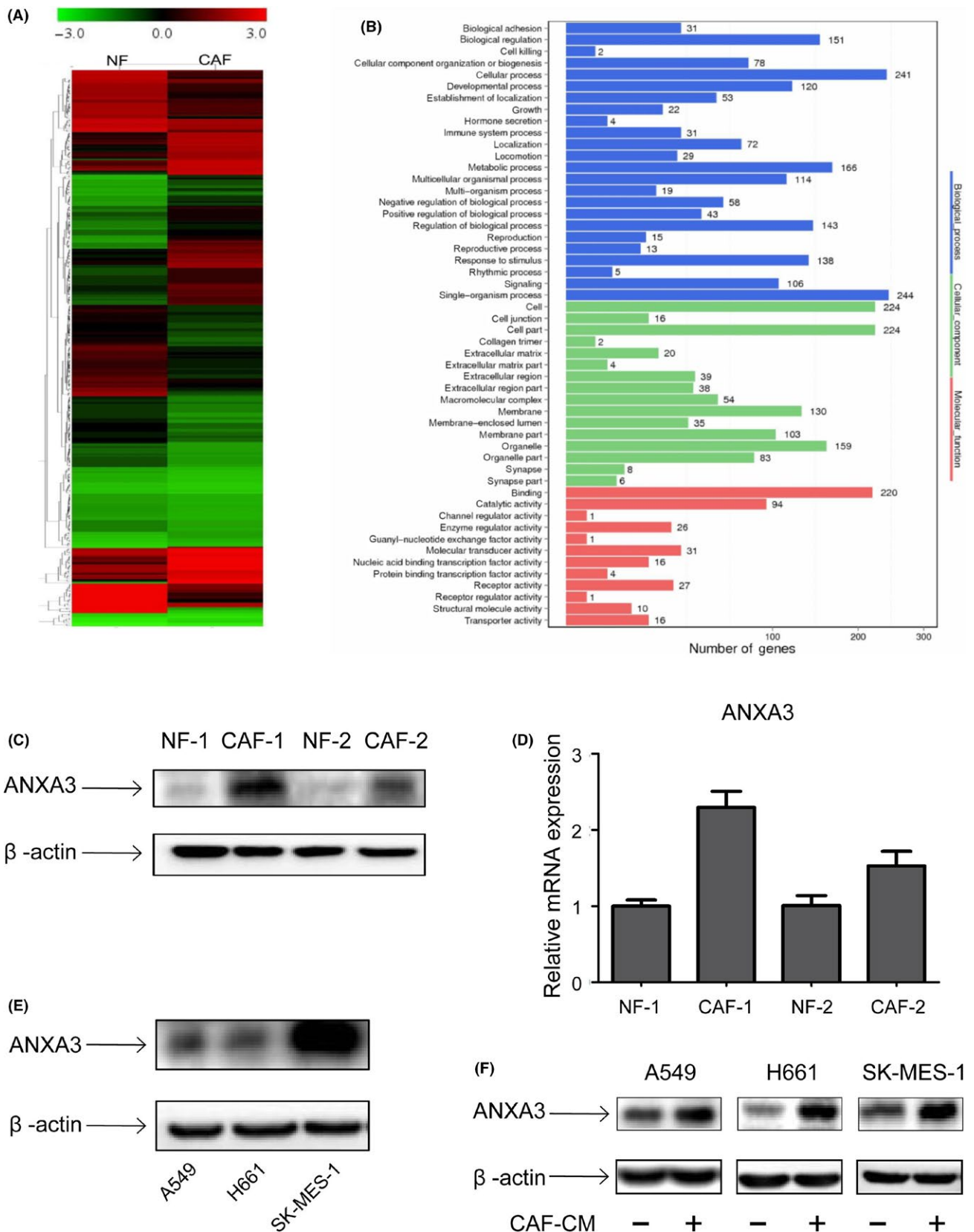
FIGURE 2 Cancer-associated fibroblasts (CAF) elevated the expression of ANXA3 in lung cancer cells. A,B, Gene expression in CAF and normal fibroblasts (NF) was assessed by RNA-Seq. The differential gene expression and the ontology analysis were performed. C, The expression of ANXA3 in paired CAF and NF were detected by western blotting. D, The mRNA expression of ANXA3 in paired CAF and NF were detected by quantitative PCR. E, The expression of ANXA3 in lung cancer cell lines was detected by western blotting. F, The expression of ANXA3 in lung cancer cells cultured with CAF-CM was detected by western blotting

6 weeks, mice were killed, and tumor tissues were dissected and used for mRNA and protein extraction.

2.11 | Statistical analysis

The data were presented as mean ± SD. The statistical analysis was performed using SPSS21.0 (SPSS, Chicago, IL, USA). Variance analysis between multiple groups was performed by one-way ANOVA. Differences between 2 groups were evaluated by Student's *t*-test. Statistical significance was defined as $P < 0.05$.

3 | RESULTS

3.1 | Cancer-associated fibroblasts enhanced cisplatin resistance of lung cancer cells

For broader interest, we chose 3 lung cancer cell lines, A549 cells (adenocarcinoma), H661 cells (large cell carcinoma) and SK-MES-1 cells (squamous cell carcinoma), which represented different pathological subtypes of lung cancer. CAF were isolated from lung cancer tumor specimens, as in our previous study,[11] and CAF-CM was collected 48 hours after culturing CAF in DMEM/F12 (1:1) medium.

To investigate the effect of CAF on cisplatin sensitivity of lung cancer cells, A549 and H661 cells were pre–incubated with

CAF-CM for 24 hours, DMEM medium and NF-CM was used as control medium. Then cells were treated with 0-80 µmol/L cisplatin for 48 hours, and cell viability was detected by CCK-8 cell viability assay. CAF-CM increased cisplatin resistance of A549, H661 and SK-MES-1 cells significantly. The IC_{50} values of cisplatin were elevated by CAF-CM, from 11.57 µmol/L (DMEM), 13.67 µmol/L (NF-CM) to 30.48 µmol/L (CAF-CM) in A549 cells; from 11.44 µmol/L (DMEM), 13.77 µmol/L (NF-CM) to 42.10 µmol/L (CAF-CM) in H661 cells; and from 15.69 µmol/L (DMEM), 19.08 µmol/L (NF-CM) to 41.06 µmol/L (CAF-CM) in SK-MES-1 cells, respectively (Figure 1). Our data indicated that CAF-CM enhanced cisplatin resistance of lung cancer cells.

3.2 | Cancer-associated fibroblasts elevated the expression level of ANXA3 in lung cancer cells

Cancer-associated fibroblasts and NF possess different characters and play different roles, based on their gene expression profiles. We compared the mRNA expression profiles between CAF and NF by applying the RNA-Sequence (RNA-Seq) approach. Our RNA-Seq data showed that 219 genes were upregulated in CAF compared to NF, whereas, 158 genes were downregulated in CAF compared to NF (Figure 2A). The gene ontology analysis classified these modulated genes into 3 categories: biological process, cellular component and molecular function (Figure 2B). In the biological process
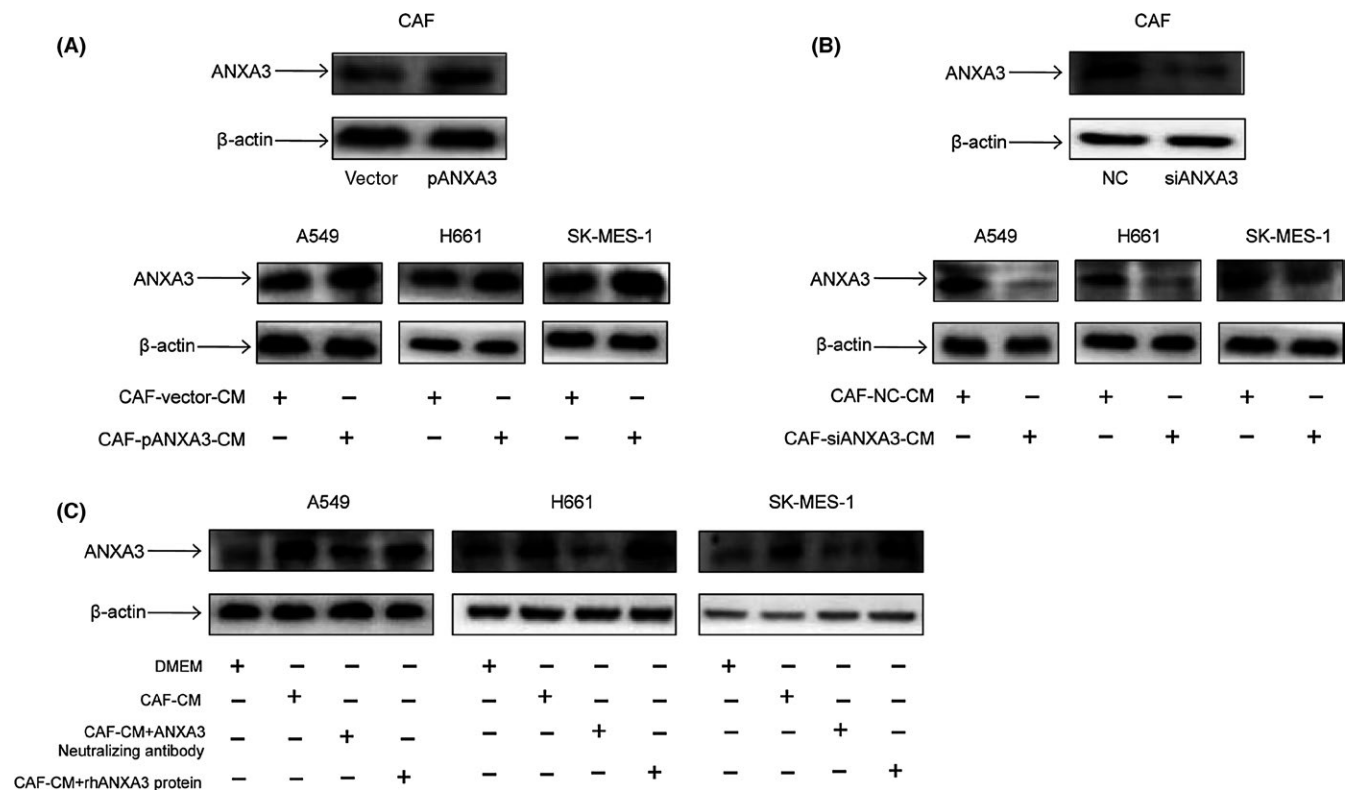

FIGURE 3 Elevated expression of ANXA3 in lung cancer cells was mediated by ANXA3 secretion in cancer-associated fibroblasts (CAF). A,B, ANXA3 was overexpressed or knocked down in CAF, the CAF-CM was collected and incubated with lung cancer cells. The expressions of ANXA3 in lung cancer cells were detected by western blotting. C, Recombinant ANXA3 was added to DMEM and incubated with lung cancer cells. The expressions of ANXA3 in lung cancer cells were detected by western blotting. ANXA3 neutralizing antibody (500 ng/mL) was added to CAF-CM and incubated with lung cancer cells. The expression of ANXA3 in lung cancer cells was detected by western blotting

category, most genes are related to cellular and single-organism processes; in the cellular component category, most genes are related to cell parts and organelles; and in the single-organism process category, most genes are related to binding and catalytic activity.

Because CAF strengthened cisplatin resistance of lung cancer cells, we focused on upregulated genes that are involved in tumor development. We found that ANXA3 is upregulated in CAF. To validate the RNA-Seq results, we performed western blot and qPCR analysis to determine the expression of ANXA3. Consistent with the results of RNA-Seq, both protein and mRNA levels of ANXA3 were significantly elevated in CAF (Figure 2C,D).

To determine whether CAF affect the expression of ANXA3 in lung cancer cells, we first examined the expression of ANXA3 in A549, H661 and SK-MES-1 cell lines. Our results showed that ANXA3 was expressed in all 3 cell lines, and the expression level of ANXA3 in SK-MES-1 cells was much higher than in A549 and H661 cells (Figure 2E). Next, we incubated A549, H661 and SK-MES-1 cells with CAF-CM, and expression of ANXA3 in lung cancer cells were determined. Figure 2F showed that CAF-CM incubation dramatically increased the ANXA3 level in all 3 lung cancer cell lines at both mRNA and protein levels.

Although CAF-CM incubation increased the ANXA3 level in lung cancer cells, we need to further demonstrate whether the increased ANXA3 level in cancer cells is due to ANXA3 secretion in CAF. We manipulated ANXA3 levels in CAF by overexpression or RNAi knock down. We found that when ANXA3 was overexpressed in CAF, its levels in lung cancer cells were increased accordingly (Figure 3A); whereas when ANXA3 level was reduced by RNAi knockdown, its levels in lung cancer cells were also decreased (Figure 3B). Furthermore, we manipulated ANXA3 levels in medium. The ANXA3 recombinant protein was added to the culture medium to mimic the ANXA3 containing CAF-CM. Figure 3C shows that ANXA3 recombinant protein enhanced ANXA3 levels in cancer cells. We further added the ANXA3 neutralizing antibody to CAF-CM, and we found that the blockage of ANXA3 secretion in CAF attenuated ANXA3 upregulation (Figure 3C). These results demonstrated that ANXA3 level increase in cancer cells via ANXA3 secretion in CAF.

3.3 | ANXA3 mediated the effect of cancer-associated fibroblasts on cisplatin resistance of lung cancer cells

To investigate the role of ANXA3 in the enhancement of CAF on chemoresistance, the expression of ANXA3 in lung cancer cells was manipulated. Because A549 and H661 cells expressed lower levels
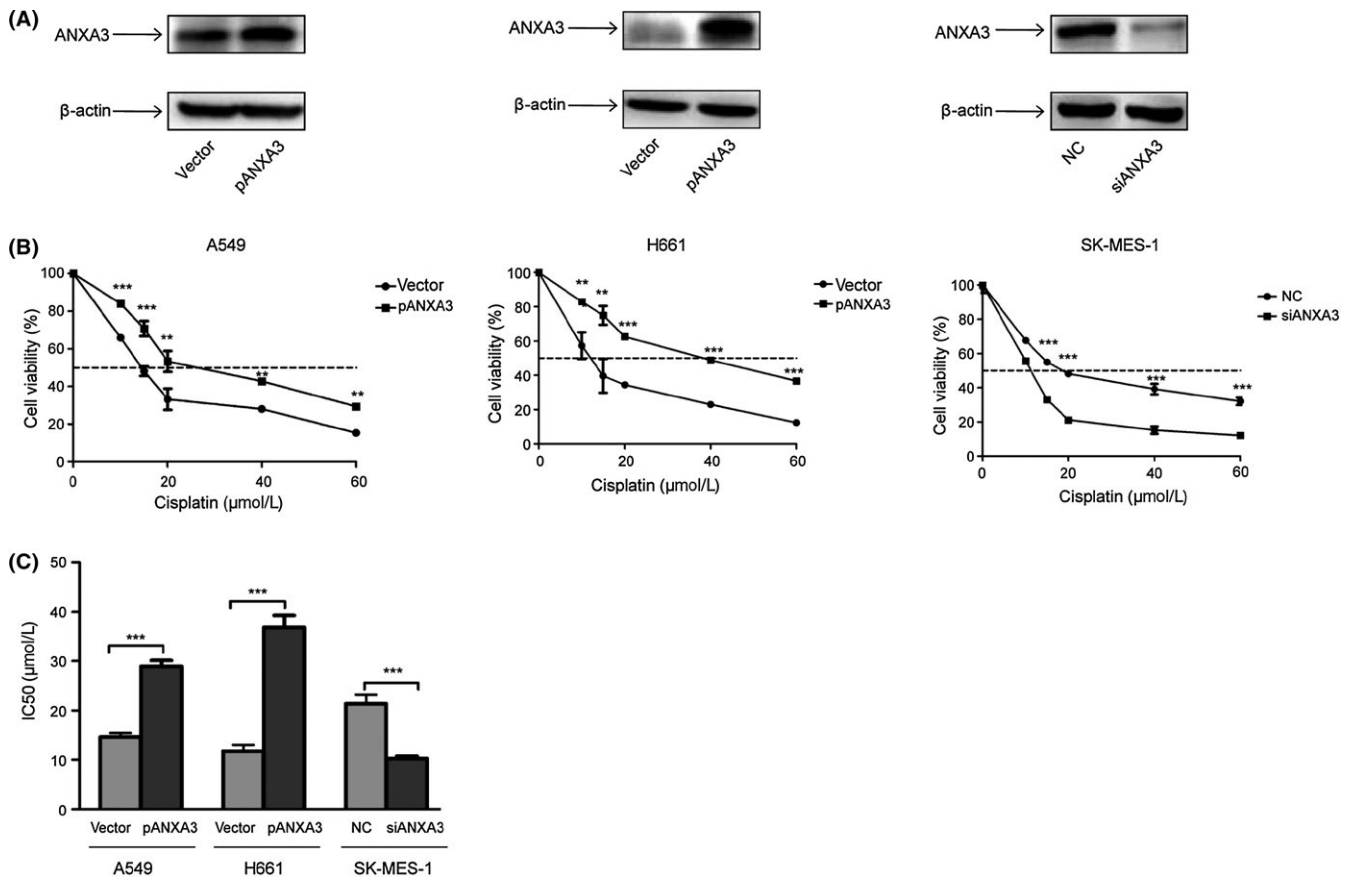

FIGURE 4 ANXA3 mediated the effect of cancer-associated fibroblasts on cisplatin resistance of lung cancer cells. A, A549 and H661 cells were transfected with pANXA3, and SK-MES-1 cells were transfected with ANXA3-siRNA duplex; ANXA3 expression were analyzed by Western blotting. B,C, After transfection cells were treated with 0-80 μmol/L cisplatin for 48 h, cell viability was detected by CCK-8 assay. Values represent the mean ± SD from 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001
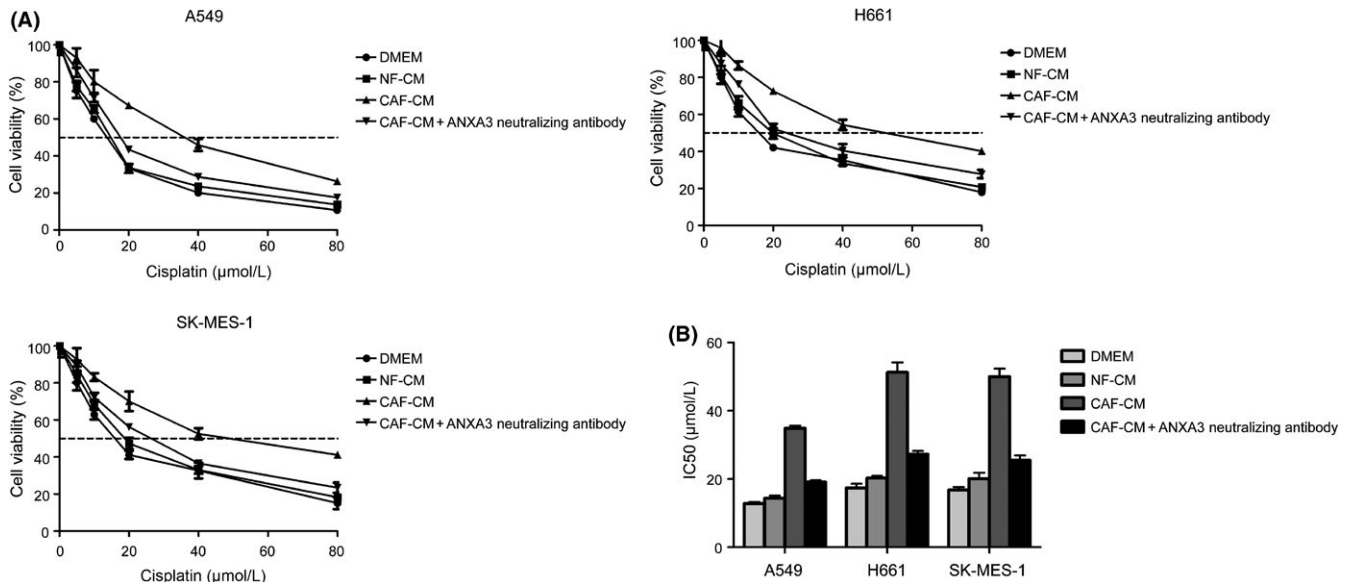
FIGURE 5 Cancer-associated fibroblasts (CAF) enhanced cisplatin resistance of lung cancer cells. Lung cancer cells were cultured in CAF-CM with ANXA3 neutralizing antibody (500 ng/mL) and treated with 0-80 μmol/L cisplatin for 48 h. Cell viability was detected by CCK-8 assay. Values represent the mean ± SD from 3 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

of ANXA3 and SK-MES-1 expressed higher levels of ANXA3, we overexpressed ANXA3 in A549 and H661 cells, whereas, knocked down ANXA3 expression in SK-MES-1 cells. Lung cancer cells were then treated with cisplatin. In Figure 4, it is evident that overexpression of ANXA3 dramatically enhanced drug resistance, IC_{50} was increased from 14.63 to 28.94 μmol/L in A549 cells, and from 11.76 to 36.83 μmol/L in H661 cells. In contrast, ANXA3 knockdown increased the sensitivity of SK-MES-1 cells; IC_{50} was decreased from 21.40 to 10.23 μmol/L. Next, we added the ANXA3 neutralizing antibody to CAF-CM, and we found that the blockage of ANXA3 secretion in CAF attenuated cisplatin resistance in cancer cells caused by CAF (Figure 5). Collectively, these results suggested that CAF potentiate cisplatin resistance by upregulating ANXA3 expression in lung cancer cells.
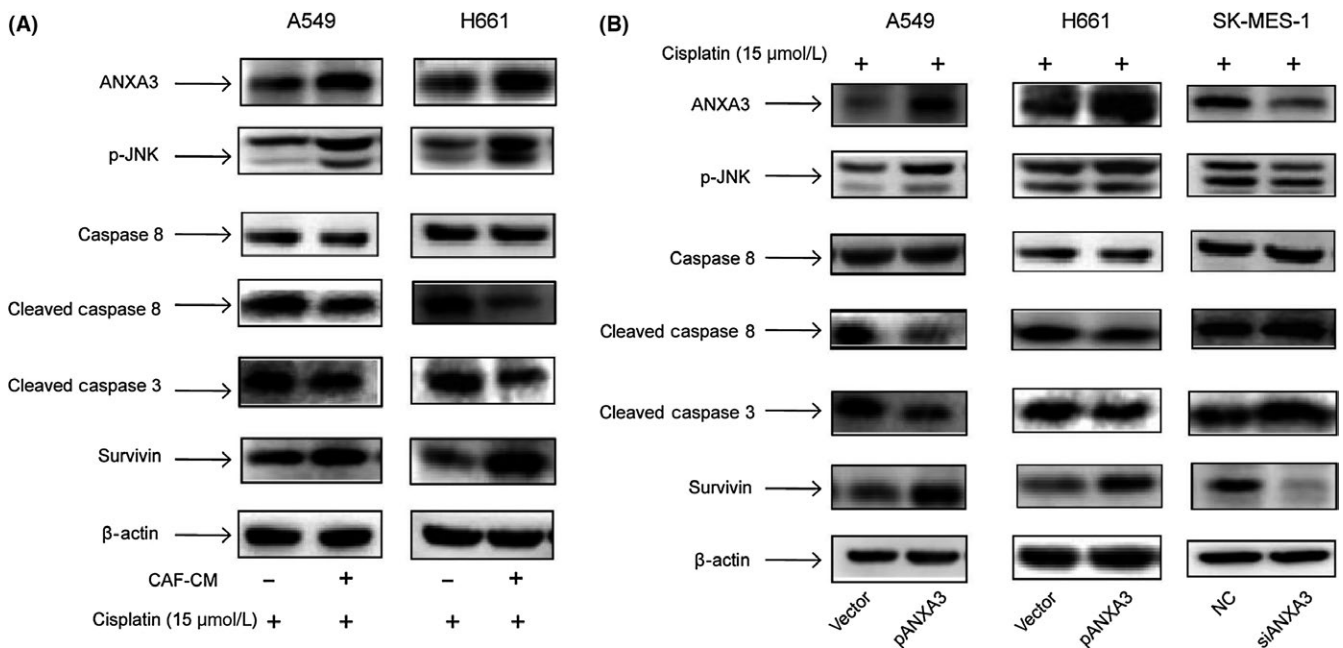

FIGURE 6 Cancer-associated fibroblasts (CAF) activated the JNK/survivin pathway by upregulating ANXA3. A, A549 and H661 cells were incubated with CAF-CM for 24 h, then treated with 15 μmol/L cisplatin for 36 h. The protein expressions were detected by western blotting. B, A549 and H661 cells were transfected with pANXA3. SK-MES-1 cells were transfected with ANXA3-siRNA duplex, then treated with 15 μmol/L cisplatin for 36 h. The protein expressions were detected by western blotting

3.4 | Cancer-associated fibroblasts activated JNK/ survivin pathway by upregulating ANXA3

Numerous studies have shown that mitogen activated-protein kinase (MAPK) signaling pathway plays an important role in cell proliferation, cell survival and drug resistance. JNK is a member of the MAPK family and is closely related to tumor development. This prompted us to explore the effect of CAF on JNK pathway.

Lung cancer cells were treated with cisplatin, with and without CAF-CM pre–treatment. Compared with cisplatin-only treatment,

CAF-CM significantly increased the expression of both ANXA3 and p-JNK. In contrast, CAF-CM decreased the expression of apoptosis-related genes cleaved caspase 8 and caspase 3, also survivin, which was activated by cisplatin (Figure 6A). We further overexpressed ANXA3 in A549 and H661 cells, then treated cells with cisplatin. We found that ANXA3 elevated the p-JNK level, and reduced the cleaved caspase-8, caspase-3 and survivin level, which had a similar effect as CAF. However, when ANXA3 expression was reduced by RNAi in SK-MES-1 cells, the JNK activation was suppressed, and the effect of cisplatin on caspases activation was reversed (Figure 6B).
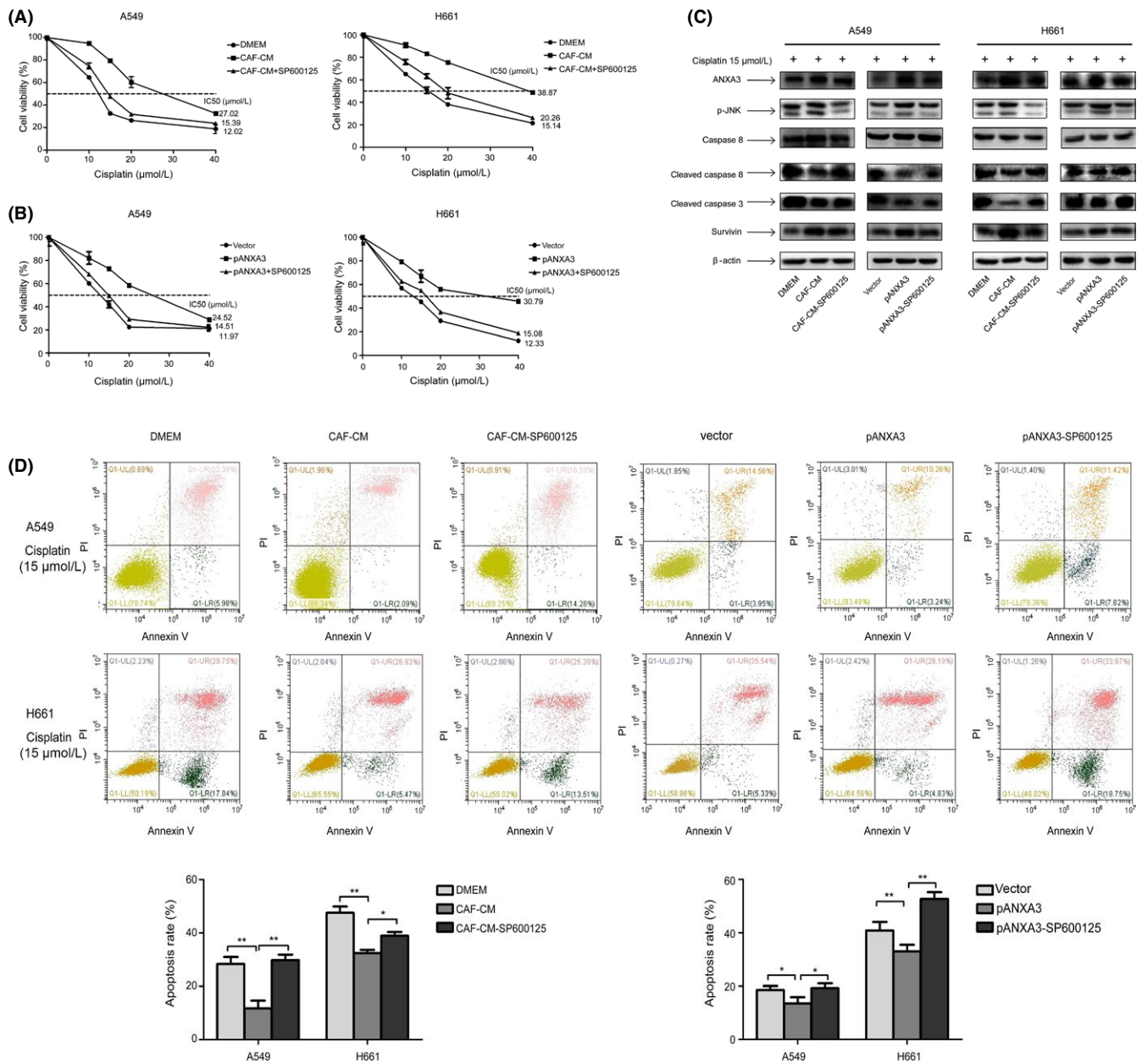

FIGURE 7 JNK/Survivin pathway mediated the effect of cancer-associated fibroblasts (CAF) on cisplatin resistance of lung cancer cells. A, A549 and H661 cells were cultured in CAF-CM and pre–treated with 2 μmol/L JNK inhibitor SP600125 for 1 h, then were treated with 0-40 μmol/L cisplatin for 48 h. Cell viability was detected by CCK-8 assay. B, A549 and H661 cells were transfected with pANXA3 and pre–treated with 2 μmol/L SP600125 for 24 h, then were treated with 0-40 μmol/L cisplatin for 48 h. Cell viability was detected by CCK-8 assay. C, Protein expressions were detected by western blotting. D, Cells were stained with Annexin/PI after treatment and the apoptotic cells were analyzed by flow cytometry. Values represent the mean ± SD from 3 independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

To further evaluate the role of the JNK/survivin signaling pathway in the effect of CAF on cisplatin resistance, we inhibited JNK activity by pretreating lung cancer cells with JNK specific inhibitor SP600125 for 1 hour, then cultured cells with CAF-CM. We found that inhibition of JNK effectively abolished the effect of CAF or ANXA3 on the cisplatin resistance (Figure 7A,B) and the activation of the JNK/survivin pathway (Figure 7C) in lung cancer cells.

Furthermore, the induction of apoptosis by cisplatin was examined by flow cytometry. Our results showed that CAF effectively protected cells from apoptosis induced by cisplatin, and inhibition of JNK by JNK specific inhibitor retarded this effect (Figure 7D). Taken together, these results demonstrated that CAF enhanced cisplatin resistance of lung cancer cells through activation of the JNK/survivin pathway via ANXA3 upregulation.

3.5 | Cancer-associated fibroblasts enhanced cisplatin resistance of lung cancer cells in vivo

To explore the effect of CAF on chemoresistance in vivo, we conducted animal experiments to evaluate the effect of CAF on tumor growth and drug resistance. A549 cells alone or combined with CAF were subcutaneously injected into mice and treated with cisplatin. For direct comparison, A549 cells alone group and A549 combined with CAF group were injected into the same mouse; A549 cells alone treated with cisplatin group and A549 plus CAF treated with cisplatin were injected into the same mouse.

Cancer-associated fibroblasts boosted tumor growth significantly in mice; the average tumor volume of A549 cells combined with the CAF group was 2.5-fold larger than for the A549 alone group after 6 weeks. As expected, cisplatin inhibited A549 cell growth in mice; interestingly, CAF dramatically enhanced tumor growth under cisplatin treatment (Figure 8A,B). These results indicated that CAF enhanced tumor growth and chemoresistance.

Next, we investigated the ANXA3 status and JNK/survivin pathway in tumors. The tumor tissues were dissected, and the ANXA3 and JNK pathway were examined by immunoblotting. Consistent with in vitro study, ANXA3 expression was increased, the level of p-JNK and survivin was also elevated. On the other hand, apoptosis-related genes caspase 3 and 8 was suppressed (Figure 8C). These results demonstrated that CAF enhance A549 xenograft growth and cisplatin resistance, through the JNK/survivin pathway activation via ANXA3.
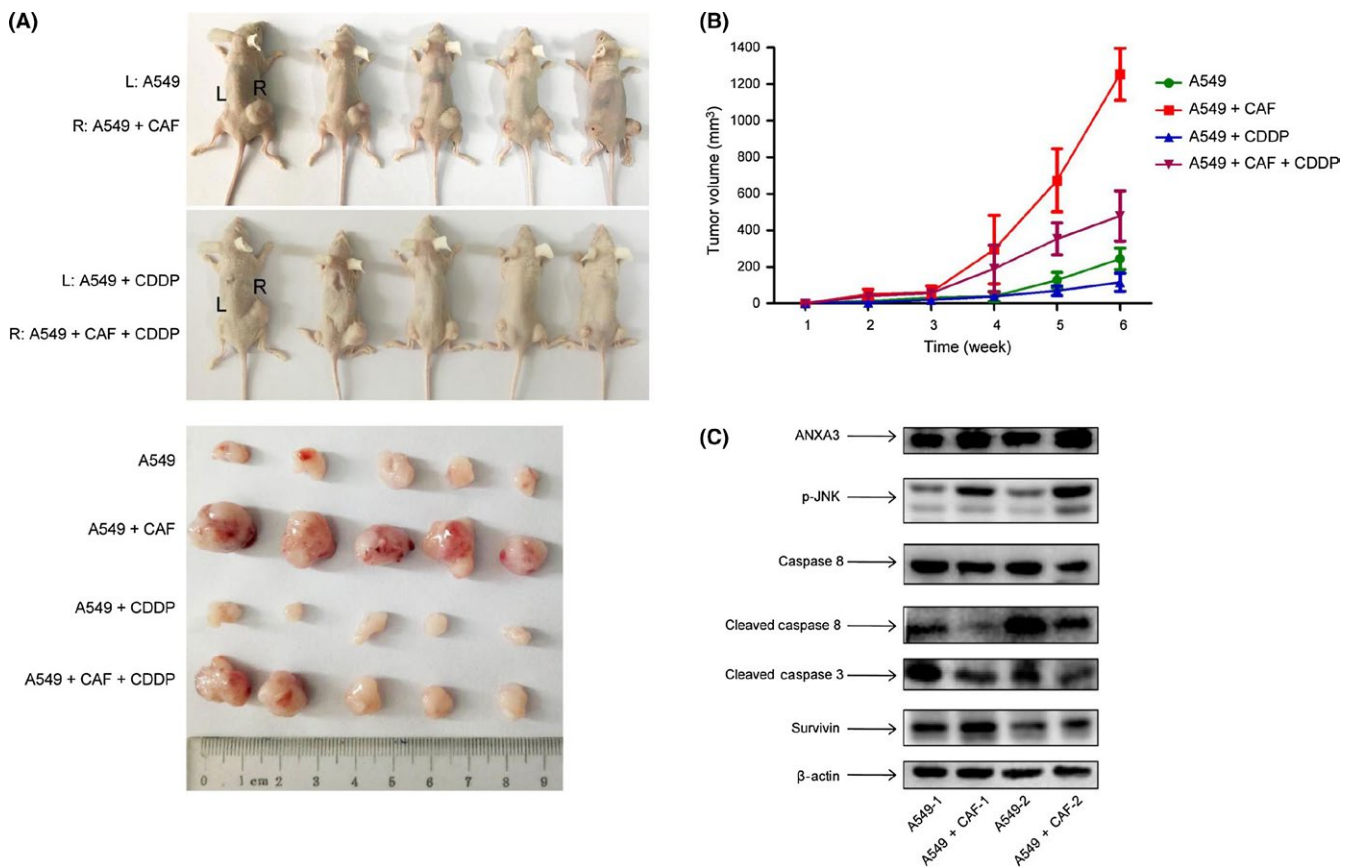

FIGURE 8 Cancer-associated fibroblasts (CAF) enhanced cisplatin resistance of lung cancer in vivo. 3×10^6 A549 cells, alone or with 9×10^6 CAF (ratio1:3) were injected subcutaneously into the back of BALB/c nude mice. Mice were treated with PBS or cisplatin (3 mg/kg body weight) for 6 wk. A, At the end of treatment, tumor formation was inspected and tumors were excised. B, Tumor volumes were measured every week. C, The protein expressions in tumor tissues were detected by western blotting
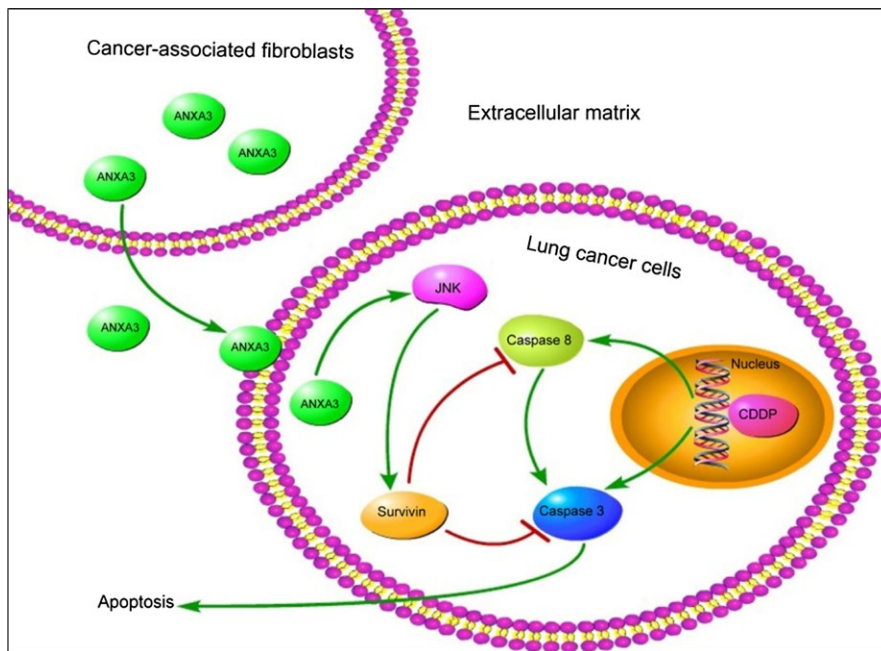
FIGURE 9 A proposed model of cancer-associated fibroblasts enhanced cisplatin resistance in lung cancer cells

4 | DISCUSSION

Drug resistance is one of the main obstacles that both clinicians and patients have to deal with during cancer treatment. Despite the extensive studies on chemoresistance, in particular, on the use of conventional chemotherapeutic reagents such as platinum compounds and antimetabolites, the specific mechanisms that cause the tumor recurrence and progression after treatment are still poorly understood.

For many years, research on drug resistance has been focused mainly on cancer cells; however, little is known about the role of TME in the response of cancer cells to chemotherapy. CAF is one of the major types of tumor stromal cells and plays crucial roles in carcinogenesis, tumor progression and metastasis.[8] In our previous study, we isolated and grew CAF from lung cancer specimens, and demonstrated that CAF promote metastasis and EMT of lung cancer cells via IL-6 secretion and JAK2/STAT3 pathway activation.[11] This finding prompted us to further investigate the role of CAF in drug resistance.

Numerous studies have shown that CAF promote drug resistance in several types of tumor, such as breast cancer,[14] ovarian cancer,[15] pancreatic cancer[16] and colorectal cancer.[17] To investigate the effect of CAF on cisplatin resistance of lung cancer, we collected the CAF-CM and cultured lung cancer cells in CAF-CM. We found that CAF-CM dramatically affects the cisplatin sensitivity of lung cancer cells.

Cancer-associated fibroblasts can secrete CAF-specific proteins, cytokines, growth factors and ECM components to support tumor cell growth, angiogenesis and confer chemoresistance.[18] To identify whether the CAF-secreted proteins contribute to drug resistance, we first performed RNA-Seq on CAF compared to NF. Among upregulated genes in CAF, ANXA3 drew our attention. ANXA3, a member of the annexin family, binds to acidic phospholipids in a calcium-dependent manner.[19] Recent studies show that ANXA3

plays an important role in tumor growth and progression. Clinical evidence indicates that ANXA3 could be a potential biomarker of several types of cancers, such as bladder cancer,[20] colon cancer[21] and gastric cancer.[22] It can also serve as an important marker for the invasion, the metastasis and the prognosis of breast cancer.[23]

ANXA3 is highly expressed in gastric cancer tissue and cell lines, and overexpression of ANXA3 promoted cell proliferation and colony formation.[22] Zhou et al[24] reported that inhibition of ANXA3 suppressed the proliferation and invasion of breast cancer cells. Interestingly, ANXA3 also involved in chemoresistance. Sorafenib-resistant hepatocellular carcinoma cells and patient-derived xenografts expressed a high level of ANXA3. Overexpression of ANXA3 in sorafenib-resistant hepatocellular carcinoma cells suppressed apoptosis, whereas anti–ANXA3 monoclonal antibody potentiated the effect of sorafenib.[25] The study by Pan et al.[26] also showed that ANXA3 promoted the resistance of hepatocellular carcinoma cells to 5-fluorouracil and cisplatin in vivo. In this study we found that the expression of ANXA3 was higher in CAF than in NF and stimulation with CAF-CM can upregulate ANXA3 expression in lung cancer cells. Overexpression of ANXA3 enhanced cisplatin resistance of A549 and H661 cells, whereas suppression of ANXA3 increased cisplatin sensitivity of SK-MES-1 cells, which suggested the important role of ANXA3 in cisplatin resistance in NSCLC cells.

The main mechanisms of cisplatin resistance include decreased drug absorption and increased drug efflux, mutations of drug targeted genes, enhanced DNA repair capacity, increased cell proliferation and activation of anti–apoptotic signaling pathways.[27] Because the ultimate goal of cisplatin and most chemotherapeutic drugs is to induce apoptosis of tumor cells, the evasion of apoptosis becomes a key feature of acquired cisplatin resistance in tumor cells.[28] Survivin is a member of the family of inhibitor of apoptosis (IAP), which is involved in the inhibition of apoptosis.[29] Inhibition of

survivin expression by a small molecule may sensitize ovarian cancer stem cells to paclitaxel,[30] silencing survivin by RNAi to promote 5-fluorouracil to eliminate colorectal cancer stem cells.[31] Lyu et al[32] report that miR-542-3p overcomes HER3 signaling-induced chemoresistance to paclitaxel by targeting survivin in HER2-overexpressing breast cancer. Our results showed that CAF-CM upregulated survivin expression, and decreased the activity of caspase 8/3, which are the key executioners of apoptosis in lung cancer cells, suggesting that CAF enhanced cisplatin resistance of NSCLC cells by inhibiting apoptosis induced by cisplatin.

To further explore the mechanism underlying the promoting effect of CAF on cisplatin resistance of lung cancer cell, we explored the signaling pathways involved in cell proliferation and survival. MAPK is an important intracellular signaling system which is responsible for transmitting various extracellular signals into the cells. It regulates a variety of physiological processes, including metabolism, survival, cell division and apoptosis.[33,34] JNK is a member of the MAPK family; it plays a controversial roles in drug resistance. Platinum continuously activates JNK to reduce drug-induced apoptosis, and inhibition of JNK activation increases the drug sensitivity of small cell lung cancer.[35] Rincon et al[36] found that inactivation of JNK by mitogen-activated protein kinase phosphatase 1 determines resistance of breast cancer cells to taxanes and anthracyclines. In contrast, Davila-Gonzalez et al[37] reported that JNK activation contributed to docetaxel response of triple-negative breast cancer (TNBC) when nitric oxide synthase (NOS) was inhibited. We found that CAF activated the JNK signaling pathway, leading to the enhanced cisplatin resistance of lung cancer cells both in vitro and in vivo.

In summary, our study showed that CAF significantly enhanced cisplatin resistance in lung cancer cells. We further demonstrated that CAF potentiated chemoresistance by suppressing cisplatin induced apoptosis by activating the ANXA3/JNK signaling pathway (Figure 9). Targeting tge ANXA3/JNK signaling pathway could be a novel strategy to overcome lung cancer resistance to cisplatin and merits further clinical evaluation.

DISCLOSURE

There are no conflicts of interest to disclose.

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