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14. ABSTRACT Recent data strongly suggest that inflammation plays a key role in emergence of tumors and metastases. I previously found that androgen ablation causes infiltration of regressing prostate tumors with immune cells, including B cells, that produce lymphotoxin which activates IKKalpha and STAT3, in prostate tumor cells that have survived hormone withdrawal, thereby accelerating the emergence of castration-resistant prostate cancer. These results suggest that the inflammatory response associated with death of the primary tumor is an important driver of castration-resistant and metastatic disease. I found that myofibroblasts activated in an autocrine way by castration-induced hypoxia, express CXCL13, which is responsible for the recruitment of B cells in the tumor remnants. Depletion of myofibroblasts results in a delay of the emergence of the castration resistant prostate cancer and in a significant reduction of the number of B cells infiltrating the tumors. I also found that a specific TGF-beta inhibitor can inhibit the activation of myofibroblasts after castration and produced a delay in the emergence of the castration resistant prostate cancer as well. These findings suggest that myofibroblasts and TGF-beta signaling are required for the recruitment of B cells in the tumor remnants and for the emergence of castration resistant prostate cancer.					
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INTRODUCTION:

I recently found that androgen ablation therapy triggers an inflammatory response that is likely to be caused by the death of androgen-dependent CaP cells. The inflammatory response results in infiltration in the tumor remnant with a variety of lymphocytes, including B cells that express lymphotoxin (LT), a member of the TNF family. LT producing B cells lead to the activation of IKK α and STAT3 in surviving CaP cells and thereby enhance the ability to survive and continue to proliferate in the absence of androgens. An unknown component of the mechanism that promotes the emergence of CR-CaP is the cell type that produces the proinflammatory chemokine CXCL13 in response to androgen ablation. CXCL13 is the chemokine that leads to the recruitment of LT-producing B cells into the tumor remnant, a process that is critical for IKK α and STAT3 activation in CaP cells and the emergence of CR-CaP. Once the CXCL13-producing cells will be identified their role in emergence of CR-CaP will be examined. This research will result in identification of new therapeutic targets for prevention of CR-CaP. For instance, we may be able to delay the emergence of CR-CaP by inhibiting the induction of CXCL13. These approaches may also be useful for inhibition of metastatic progression of CaP.

BODY:

We have already shown (Ammirante et al., 2010) that castration-induced cell death of androgen-dependent CaP triggers an inflammatory response in the tumor remnants that recruits several immune cells in the tumor. If we castrate wild type mice and we analyze the prostate, we can find a similar expression of inflammatory cytokines and recruitment of immune cells as seen in the CaP tumors (Fig 1A, B).

Interestingly we have found activated fibroblasts and expression of CXCL13, the chemokine that recruits B cells (Ammirante et al., 2010) only in tumor from castrated mice (Fig 2). CXCL13 expression in the tumor remnants after castration appears to be localized to fibroblasts (Fig 3A) and not to CD11b⁺ or CD11c⁺ cells (Fig 3 B). To study the role of activated fibroblasts in the emergence of CR-CaP we used a DNA vaccine to ablate FAP⁺ cells (Liao et al, 2009). We observed a delay in the regrowth of CR-CaP tumors in tumor-bearing mice in which FAP⁺ cells are deleted (Fig 4 A, B). More importantly deletion of FAP⁺ fibroblasts results in a strong reduction of infiltrating B cells and other immune cells (Fig 5 A), in lower expression of several chemokines, in particular CXCL13 (Fig 5 B, C) and in absence of IKK α nuclear translocation (Fig 5 D). These results indicate that fibroblasts might be the stromal source of CXCL13, the chemokine that recruits B cells in the tumor remnants.

During the emergence of CR-CaP tumors we have observed an increase in TGF- β expression starting at day 4 after castration (Fig 6 A). TGF- β stimulation of fibroblasts purified from castrated mice (1 week after castration) can induce in vitro the induction of CXCL13 (Fig 6 B) and can activate its promoter (Fig 6 C). Although we couldn't find any SMAD responsive elements on CXCL13 promoter, we speculate that probably TGF- β can regulate the expression of CXCL13 probably through an indirect mechanism. Mice treated ED with SB-431542, a specific TGF- β inhibitor, showed a delay in the regrowth of CR-CaP (Fig 7), a reduction in B cell infiltration and CXCL13 expression, lack of fibroblasts activation, reduction of the infiltration of immune cells (Fig 8-9 A) and change in expression of CXCL13 and other chemokines (Fig 9 B, C, D, 10 A). TGF- β inhibitor is able to block the activation of SMAD4 and the activation of fibroblasts and nuclear localization of IKK α after castration (Fig 10 B, C). The source of TGF- β appears to be activated fibroblasts from the tumor stroma but not epithelial cells (Fig 11 A) or CD11b⁺ and CD11c⁺ cells (Fig 11 B). After castration we have observed also an increase in the expression of IGF-1, GP38 and CTGF (Fig 12 A). When we deleted the activated fibroblasts with the FAP DNA vaccine, the expression of IGF-1, CTGF and TGF- β dramatically decreased (Fig 12 B), indicating that myofibroblasts most probably are the source of all these soluble factors. In vitro inactive fibroblasts purified from uncastrated CaP tumor-bearing mice can be differentiated into myofibroblasts by stimulation of either CTGF + TGF β or IGF-1 (Fig 13 A, B). Interestingly incubation of inactive fibroblasts in a hypoxic chamber for 24 hrs can induce the expression of CTGF, TGF β and IGF-1 and can differentiate the fibroblasts into myofibroblasts (Fig 14). These results together demonstrate that hypoxia can induce the expression of soluble factors (i.e. CTGF, TGF β and IGF-1) that activate fibroblasts itself in an autocrine way, and once differentiated into myofibroblasts can express CXCL13 in a SMAD4-dependent fashion.

KEY RESEARCH ACCOMPLISHMENTS

hypoxia activates myofibroblasts, TGF- β activates myofibroblasts, CXCL13 is expressed by myofibroblasts.

REPORTABLE OUTCOMES

N/A

CONCLUSION

The results obtained in the first year of training grant show that fibroblasts are activated by castration-induced hypoxia in an autocrine way through the production of TGF- β , which induces the expression of CXCL13. The inhibition of TGF-beta or the development or the use of neutralizing antibodies against CXCL13 could be used as possible therapy to delay the emergence of CR-CaP

REFERENCES

Ammirante M, Luo J, Grivennikov S, Nedospasov SA, Karin M. B cell-derived lymphotoxin promotes castration-resistant prostate cancer. *Nature*. 2010;464(7286):302-5

Legend to the Figures:

Figure 1: Six weeks old FVB mice (n=10) were inoculated with myc-CaP cells and when tumors reached 1000 mm³, were castrated or sham operated. Tumors were collected at 1 week after castration for analysis. Total RNA was isolated from tumor samples of above mice and expression of the indicated cell marker mRNAs was quantitated and normalized to that of cyclophilin A (C1= mice analyzed 1 week after castration, sham= sham-operated). Results are averages $\pm$ s.d. (n=3).

Figure 2: Myc-CaP tumors were established as described above (n=10). When tumors reached 1000 mm³, mice were castrated. Tumors were analyzed one week after operation for presence of α -SMA and FSP-1 by immunohistochemistry and for expression of CXCL13 by *in situ* hybridization.

Figure 3: Myc-CaP tumors were established as above (n=10). Tumors were collected one week after castration, and fibroblasts, epithelial (A, B), CD11b⁺ and CD11c⁺ fraction (B, C) was separated. Total RNA was isolated from cell fractions and expression of the indicated mRNAs was quantitated and normalized to that of cyclophilin A.

Figure 4: 6-weeks old male FVB mice were vaccinated with 10⁸ CFU of TOPO or FAP vaccines every 5 days for three times. Myc-CaP tumors were established (n=10 per group). Three days before castration another dose of vaccine was administered. **A.** Tumor volume was measured every 2-3 days. **B.** One week after castration, tumors were collected, paraffin-embedded, sectioned and stained with α -SMA antibody.

Figure 5: FVB mice were vaccinated with TOPO and FAP vaccine as above and myc-CaP tumors were established. One week after castration tumors were collected. **A, B.** Total mRNA was isolated and expression of the indicated cell markers or chemokines mRNAs were quantitated and normalized to cyclophilin A mRNA. **C, D.** Paraffin-embedded tumor sections were stained with α -SMA, CXCL13 and IKK α antibodies by immunohistochemistry.

Figure 6: **A.** Myc-CaP tumors (n=10 per group) were established as above and tumors were collected 2, 4, 6, 7 days after castration. Total RNA was isolated and TGF- β 1, TGF- β 2 and TGF- β 3 mRNAs were quantitated by Q-PCR and normalized to the amount of cyclophilin A mRNA. **B.** Myofibroblast were purified from myc-CaP tumors 1 week after castration and stimulated for 4 hrs with hTGF- β 1 (10 ng/ml). RNA was isolated and analyzed for expression of the CXCL13 mRNA as described above. **C.** CXCL13 (+12 to -688) promoter region was cloned upstream to a luciferase reporter (pGL3-Basic vector) and 293 cells were transiently transfected with this construct. Transfected cells were stimulated with hTGF- β 1 (10 ng/ml) for 15 min and luciferase activity was measured. The results are averages $\pm$ s.d. of three independent experiments normalized to renilla activity, produced by a co-transfected renilla expression vector.

Figure 7: FVB mice bearing myc-CaP tumors were castrated. Starting one day before castration, the mice (n=10 per group) were injected daily with vehicle or SB-431542 (0.2 mg/mouse), an inhibitor of STAT3 phosphorylation. Tumor volume was measured every 2-3 days.

Figure 8: FVB mice (n=10 per group) bearing myc-CaP tumors were castrated and treated with SB-431542 as above. **A.** Tumors were collected 1 week after castration and the indicated mRNAs were quantitated. **B.** Tumors were collected 1 week after castration and the fibroblast and epithelial fraction was separated. RNA was isolated and the indicated mRNAs were analyzed by Q-PCR. **C.** Mice were sacrificed and tumors, liver, lung, kidney and prostate were collected, RNA was isolated and α -SMA mRNA was quantitated. **D.** Tumors were collected 1 week after operation, paraffin-embedded and stained with α -SMA antibody by immunohistochemistry. **E.** Tumors were collected 2, 4 6 and 7 days after castration, RNA was obtained and FAP, B220 and CXCL13 mRNAs were quantitated.

Figure 9: FVB mice (n=10 per group) bearing myc-CaP tumors were castrated and treated with SB-431542 as above. **A.** Tumors were collected 1 week after castration, total RNA was isolated and the indicated cell marker mRNAs were measured. **B, C.** Fibroblasts were isolated from tumors 1 week after castration, total RNA was isolated and the indicated chemokine mRNAs were measured. **D.** Tumors were collected 1 week after castration, total RNA was isolated and the indicated chemokine mRNAs were measured.

Figure 10: A, B, C. FVB mice (n=10 per group) bearing myc-CaP tumors were castrated and treated with SB-431542. Tumors were collected 1 week after operation, paraffin-embedded and analyzed with α -SMA, CXCL13, SMAD2/3 and IKK α antibodies by immunohistochemistry.

Figure 11 A, B: FVB mice (n=10 per group) bearing myc-CaP tumors were castrated and sacrificed 1 week after castration. Fibroblasts, epithelial cells, CD11c⁺ and CD11c⁺ cells were separated, total RNA was obtained and TGF- β 1, TGF- β 2 and TGF- β 3 mRNAs were quantitated by Q-PCR.

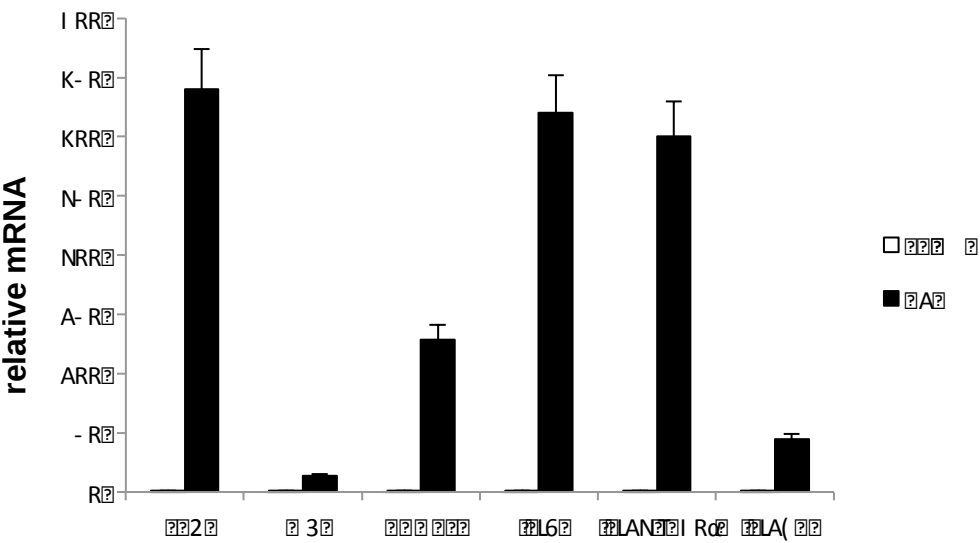
Figure 12: A. Myc-CaP tumors (n=10 per group) were established and tumors were collected 2, 4, 6, 7 days after castration. Total RNA was isolated and CTGF, GP38 and IFG-1 mRNAs were quantitated. **B.** FVB mice were vaccinated with TOPO and FAP vaccine and myc-CaP tumors were established. One week after operation tumors were collected and the indicated cytokine mRNAs were quantitated.

Figure 13 A, B: Myofibroblasts were purified from tumor-bearing myc-CaP tumors 1 week after castration, and stimulated for 24 hrs with the indicated cytokines (hTGF- β 1: 10ng/ml, CTGF: 50ng/ml, IFG-1: 100ng/ml). At the end of the experiment the myofibroblasts were collected, total RNA was isolated and α -SMA, FAP and CXCL13 mRNAs were quantitated.

Figure 14: Myofibroblasts were purified as above 1 week after castration and incubated or not in an hypoxic chamber for 24 hrs. Total RNA was isolated and CTGF, IGF-1, TGF- β 1, TGF- β 2, TGF- β 3, α -SMA, FAP and CXCL13 mRNAs were quantitated.

Figure 1

A



B

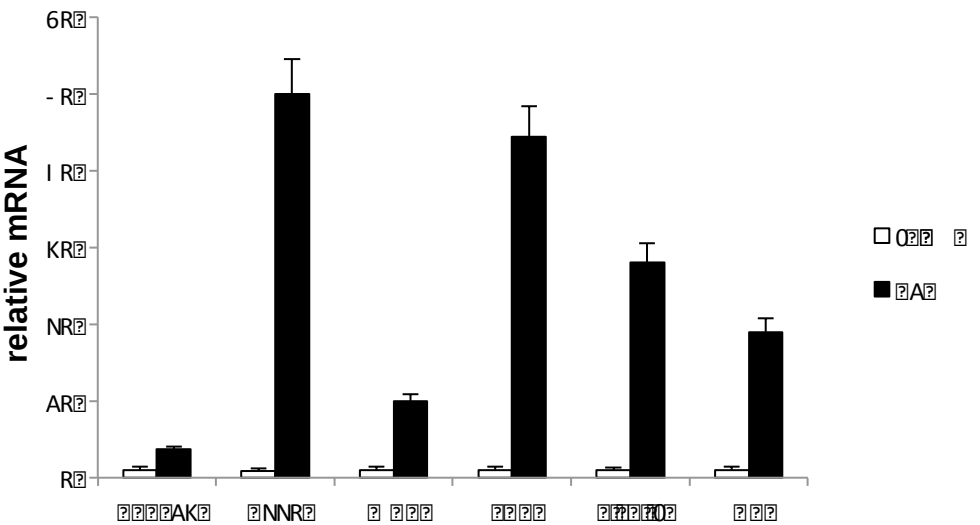


Figure 2

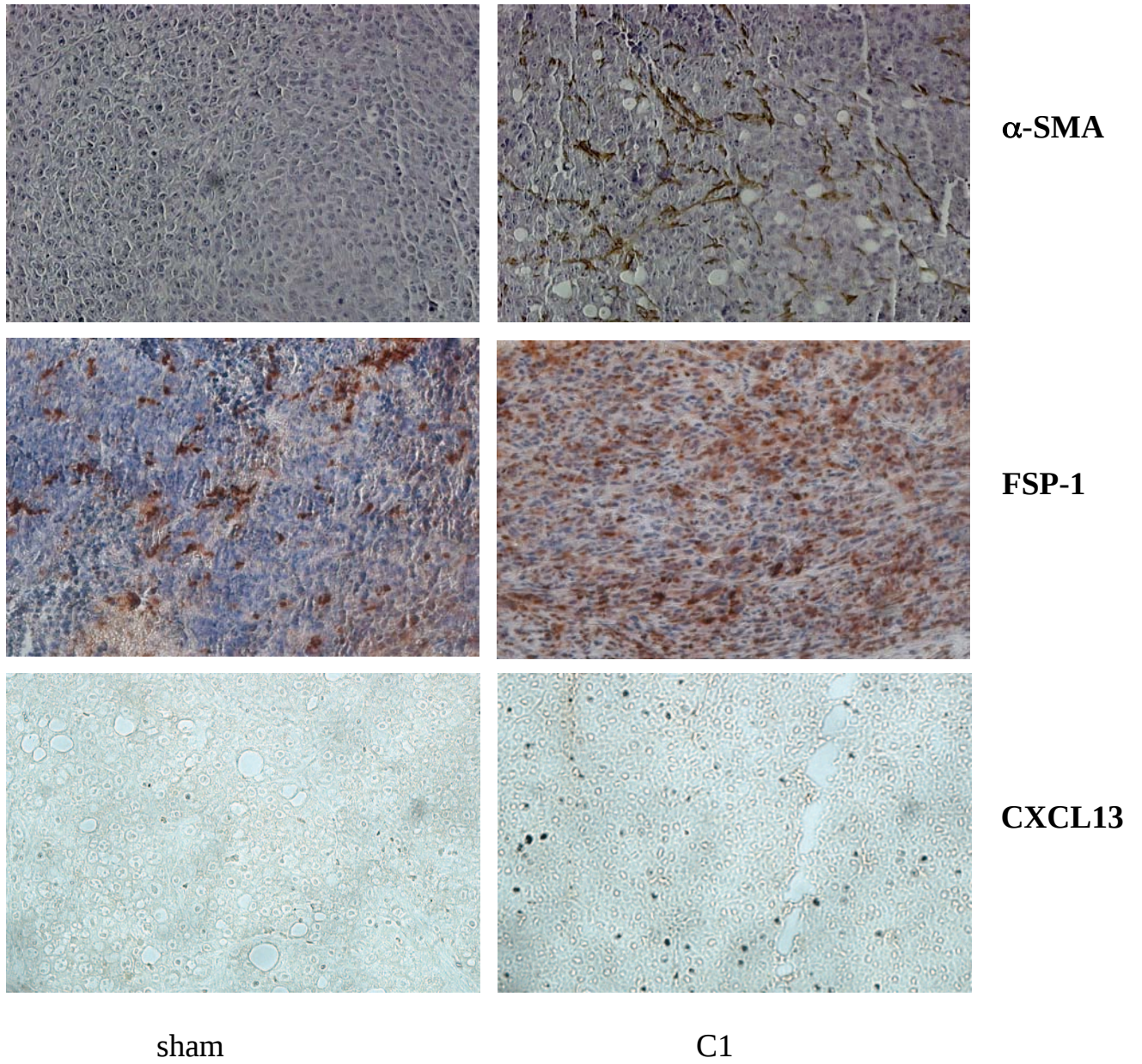


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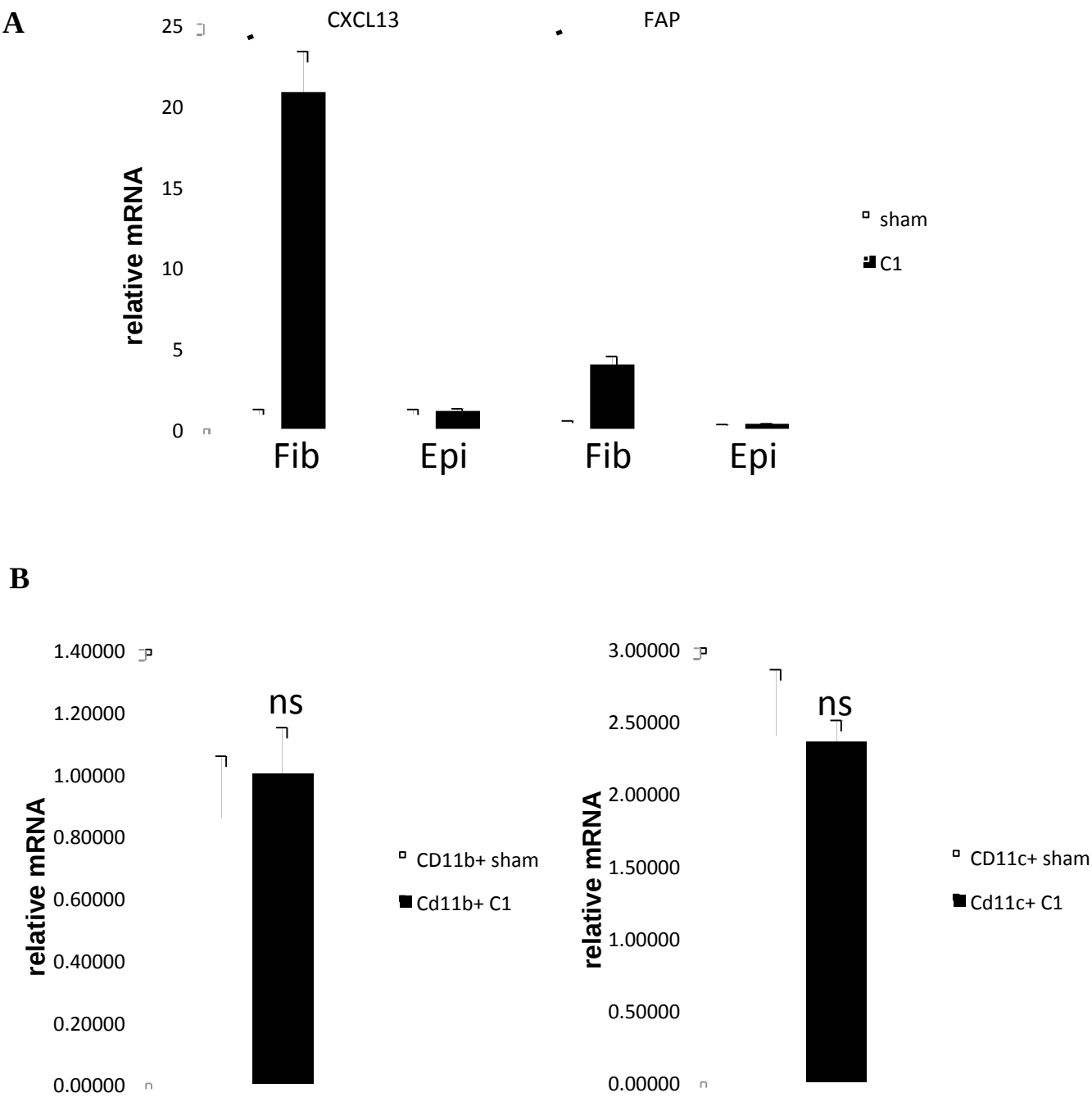
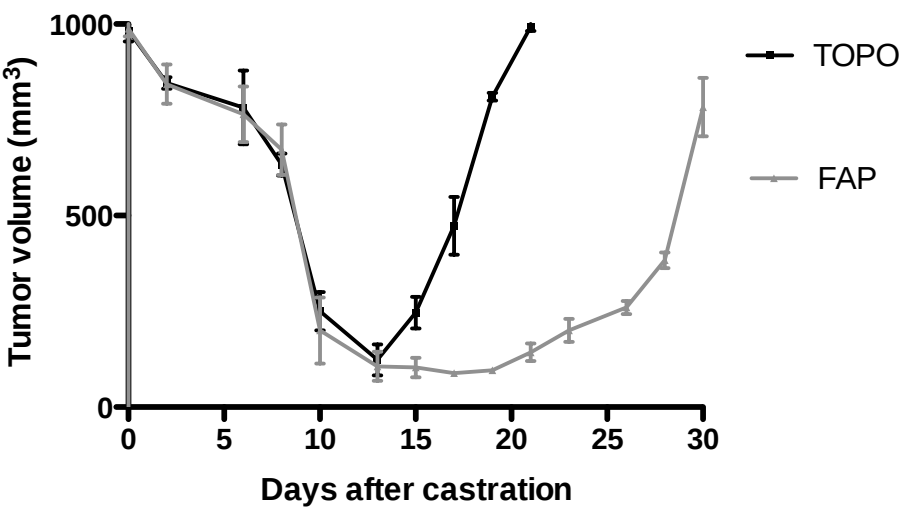


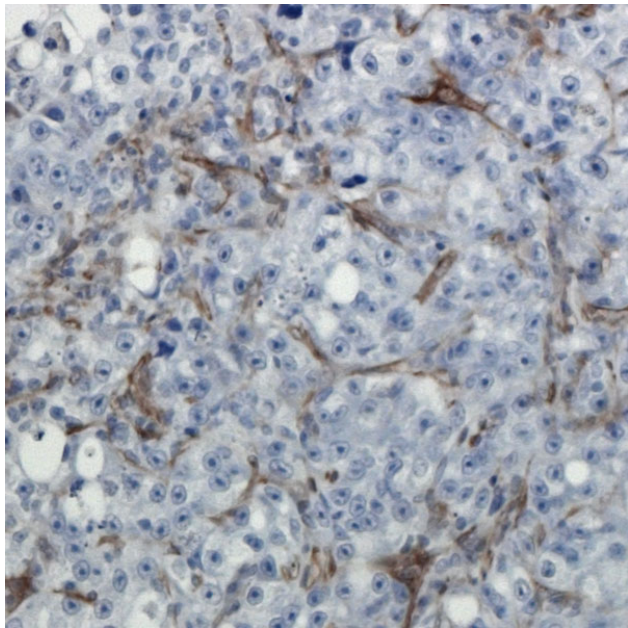
Figure 4

A

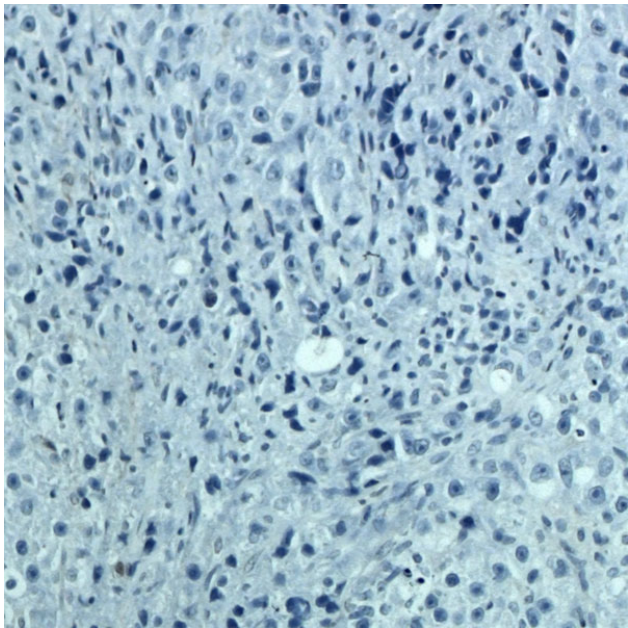


B

20X



TOPO



FAP

α -SMA

Figure 5

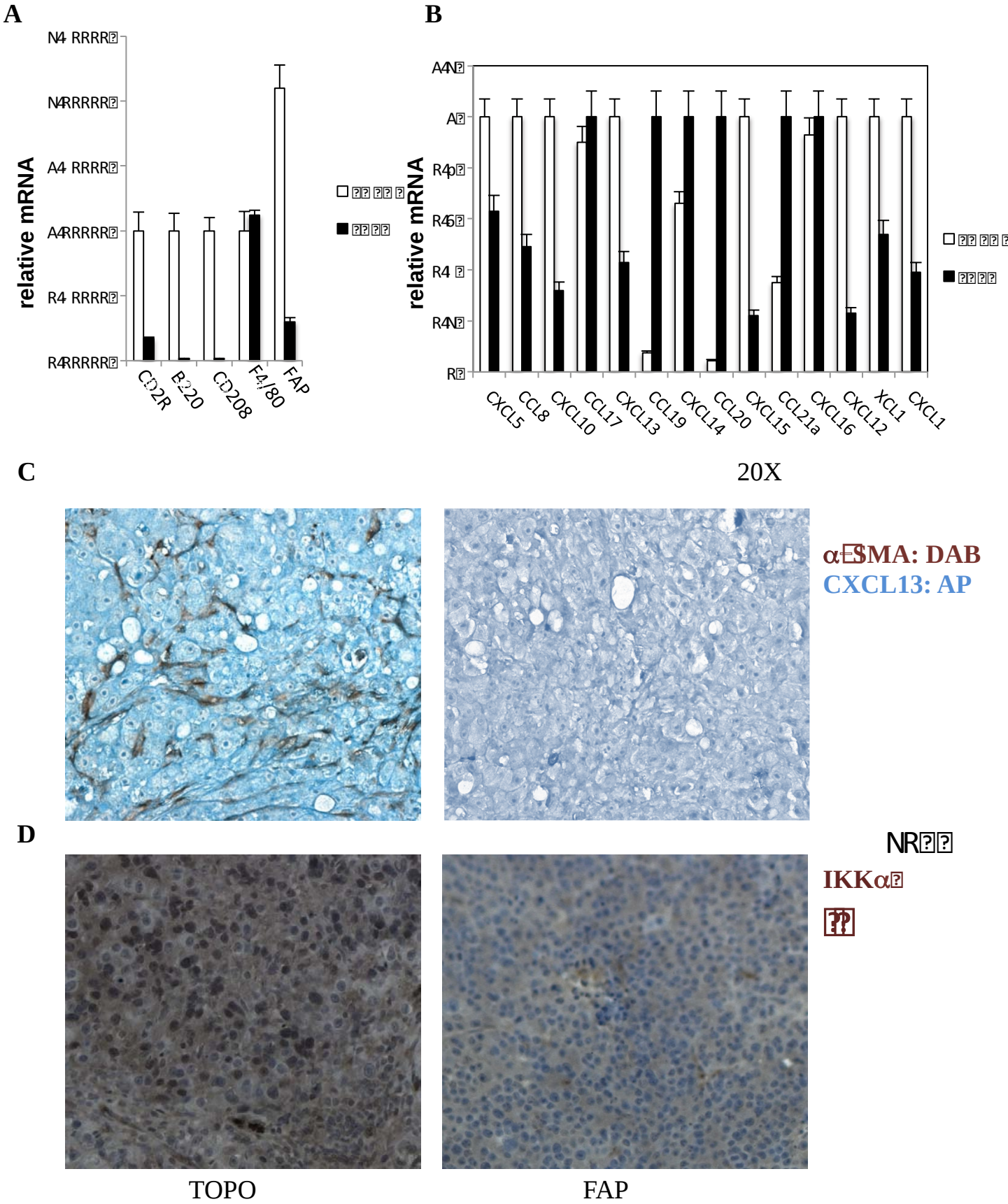
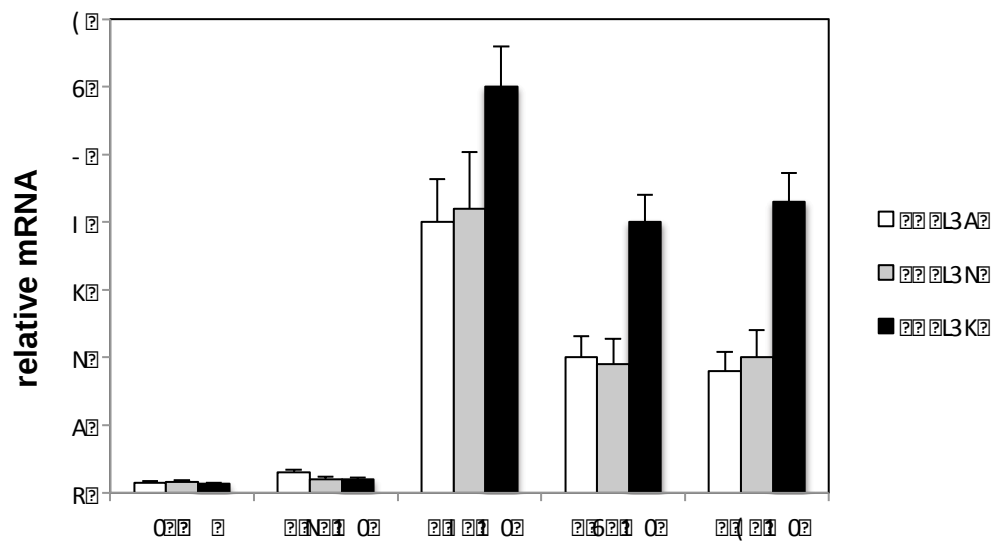
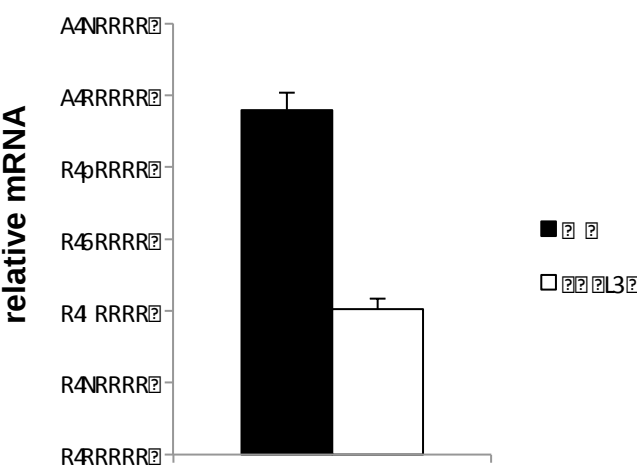


Figure 6

A



B



C

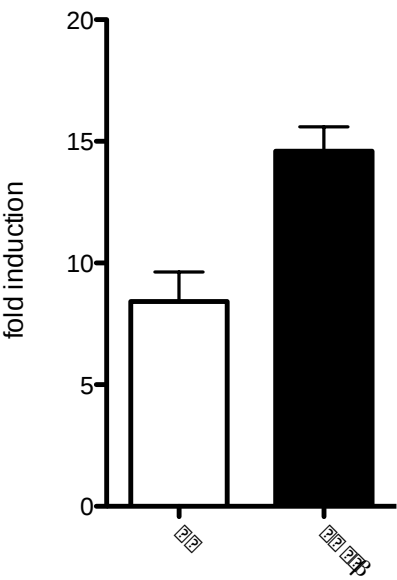


Figure 7

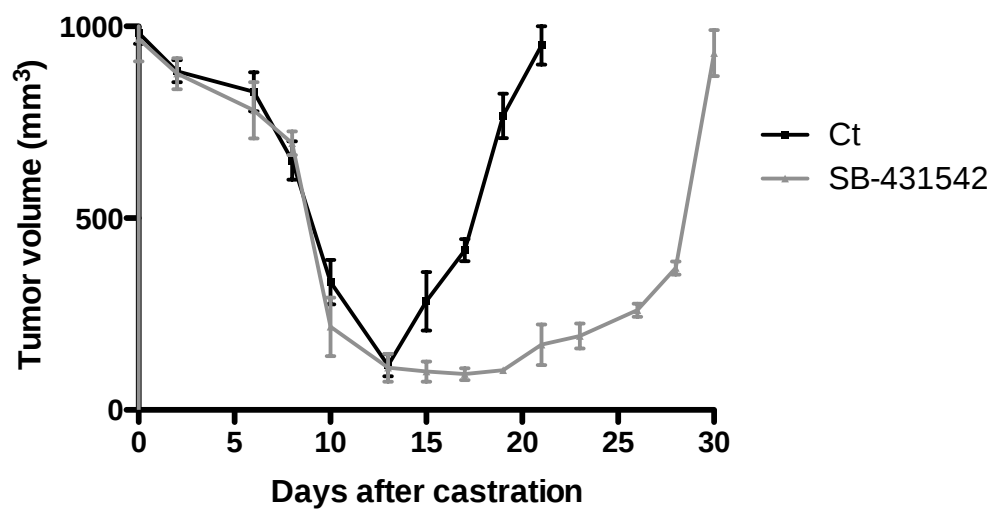


Figure 8

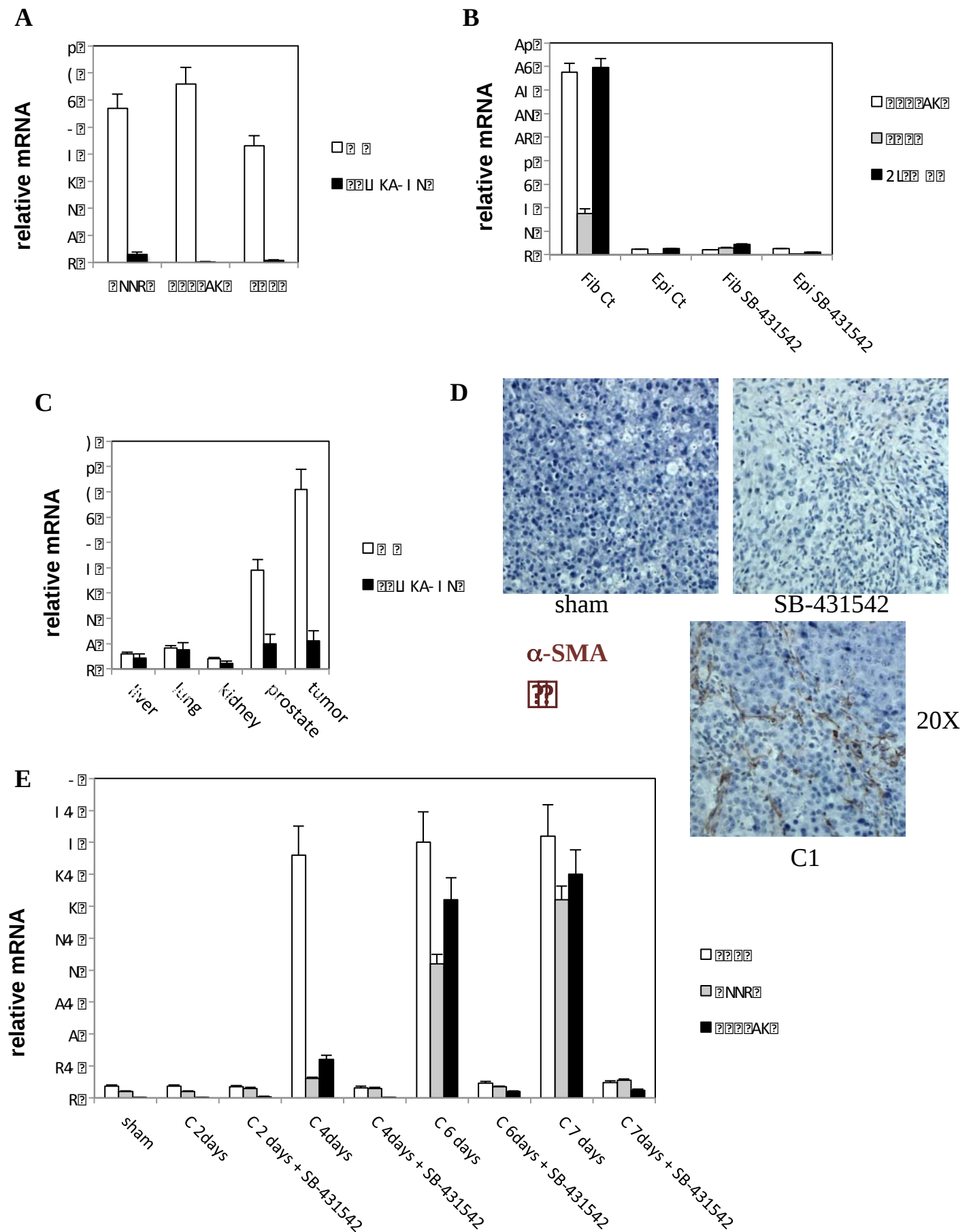


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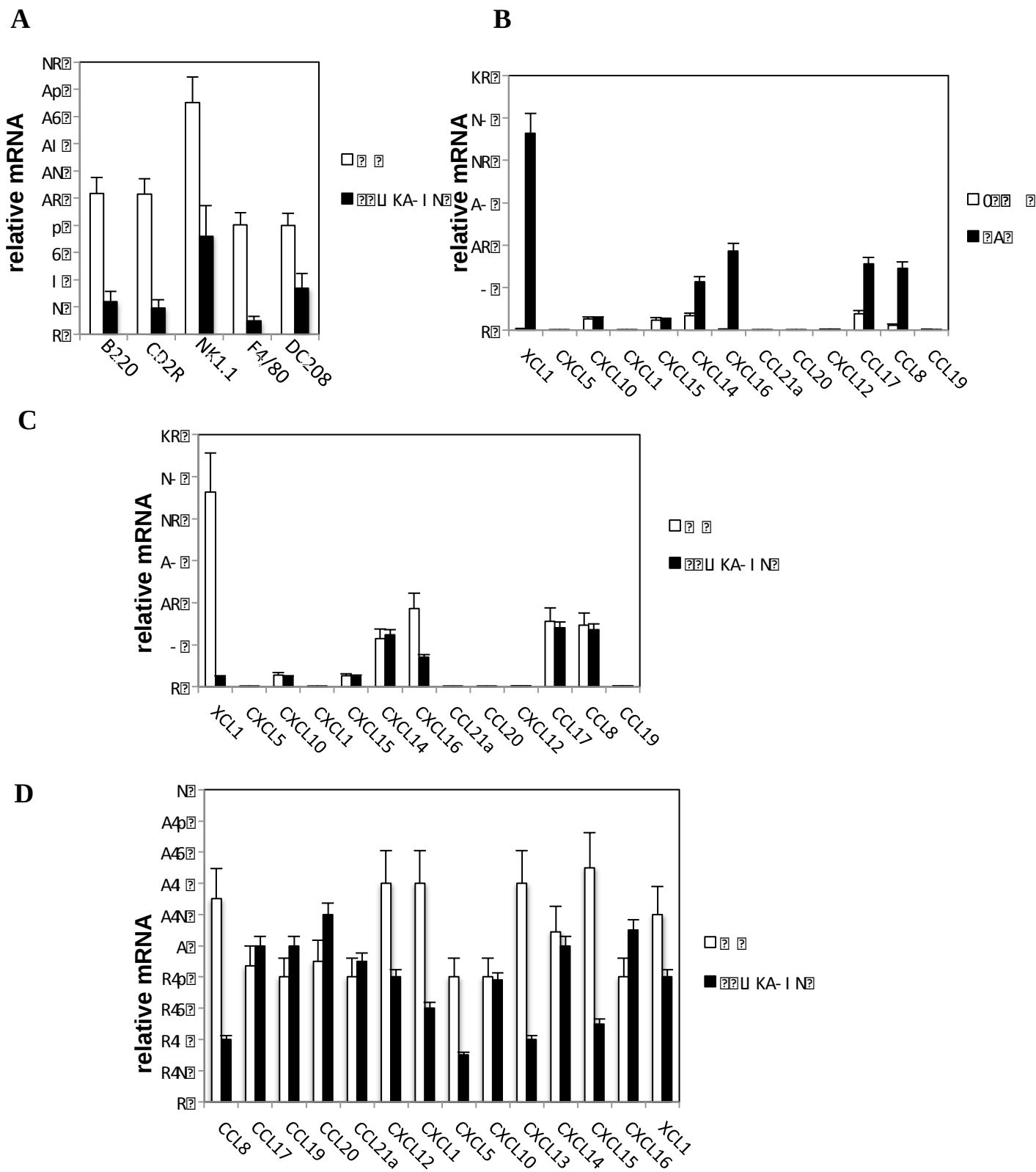
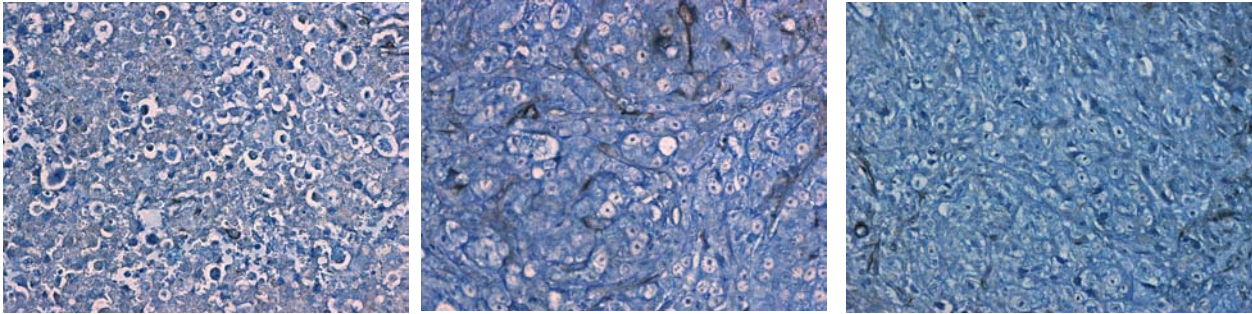


Figure 10

α -SMA: DAB
CXCL13: AP

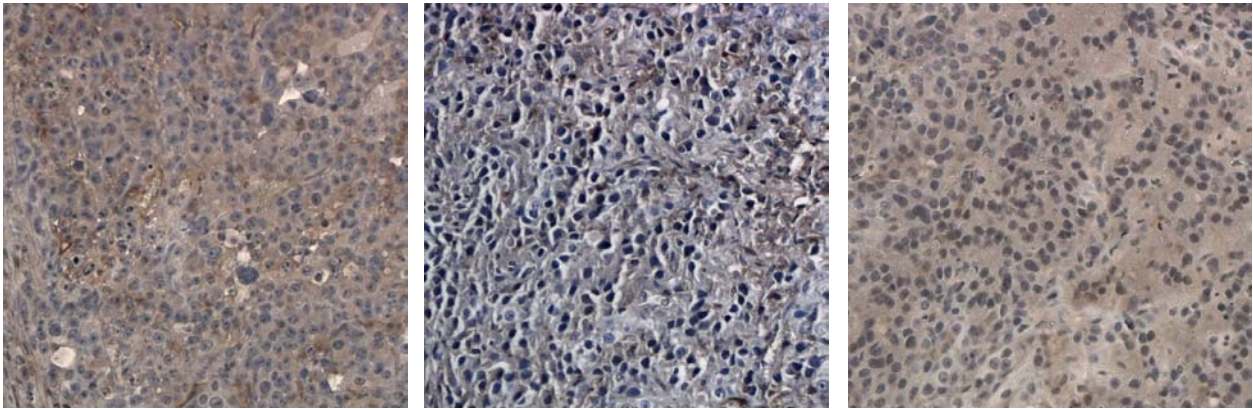
A



20X

B

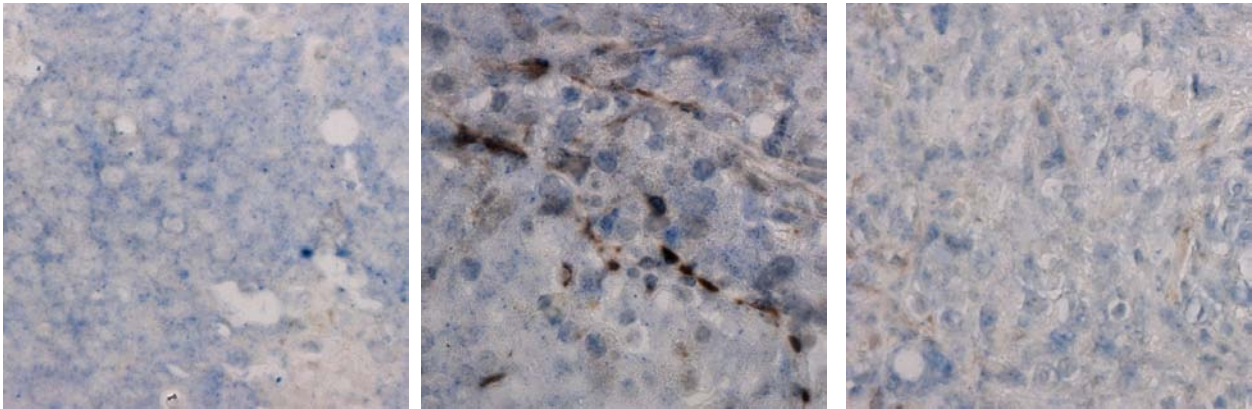
IKK α



20X

C

α -SMA: DAB
SMAD: AP



40X

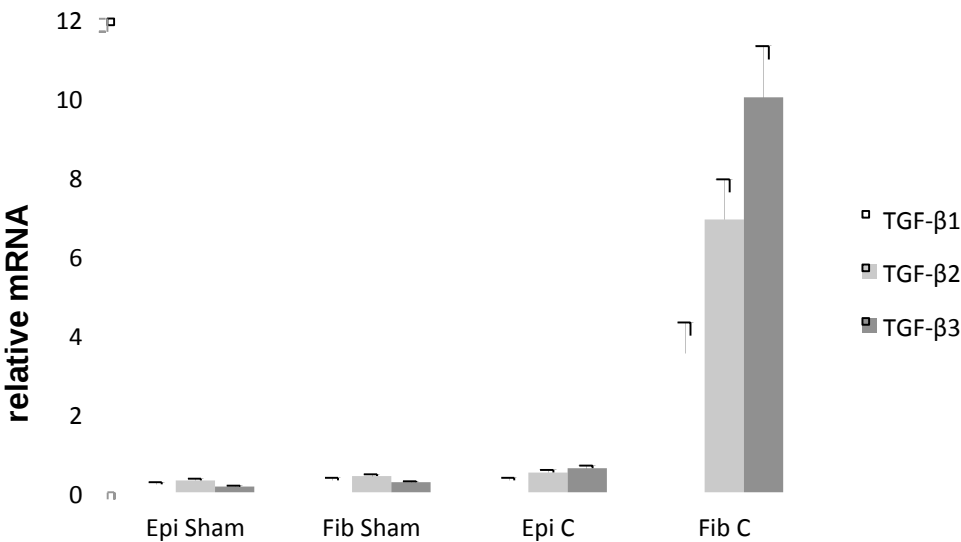
Sham

C1

SB-431542

Figure 11

A



B

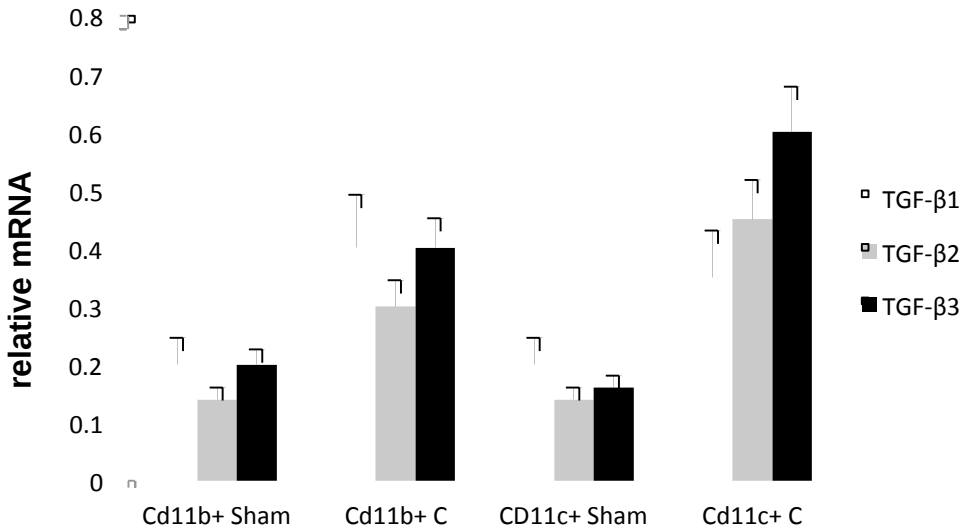
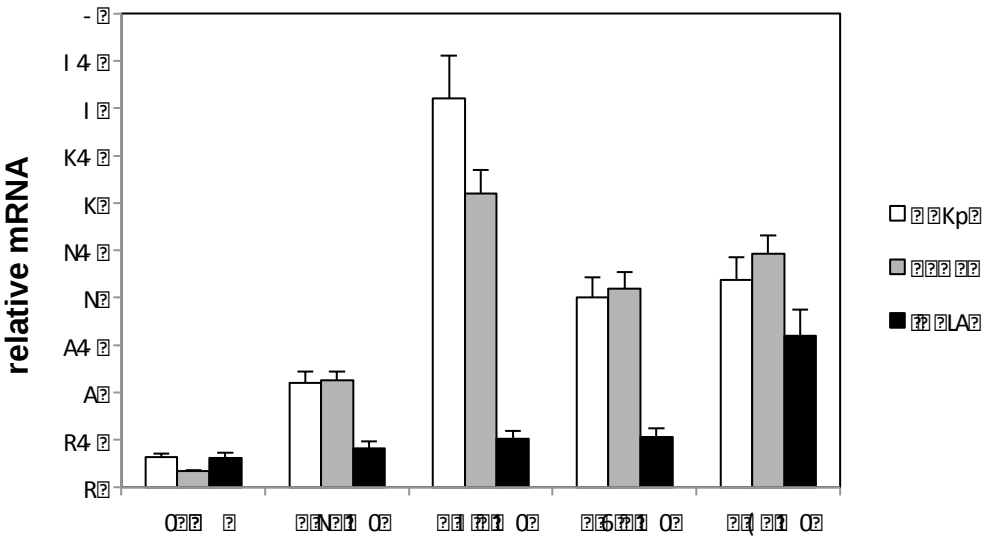


Figure 12

A



B

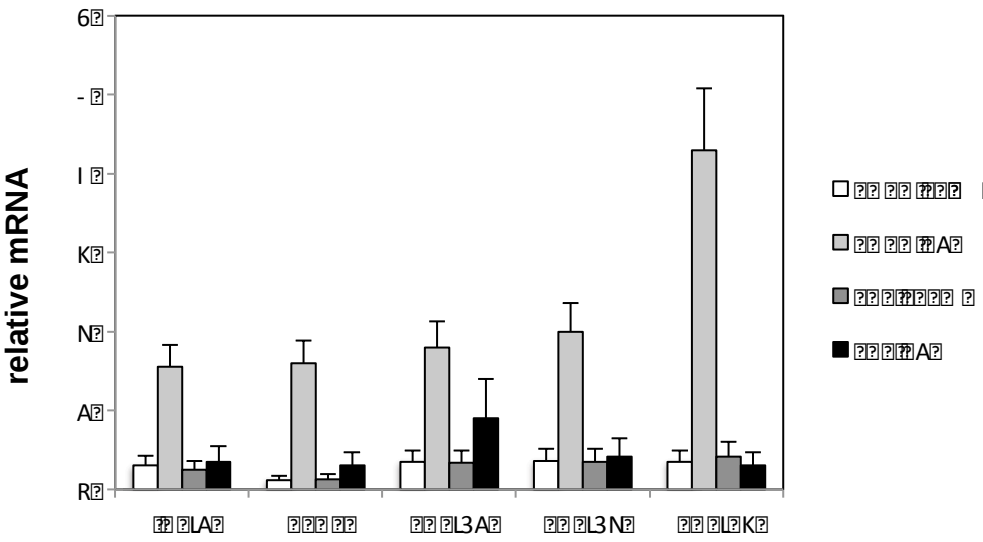
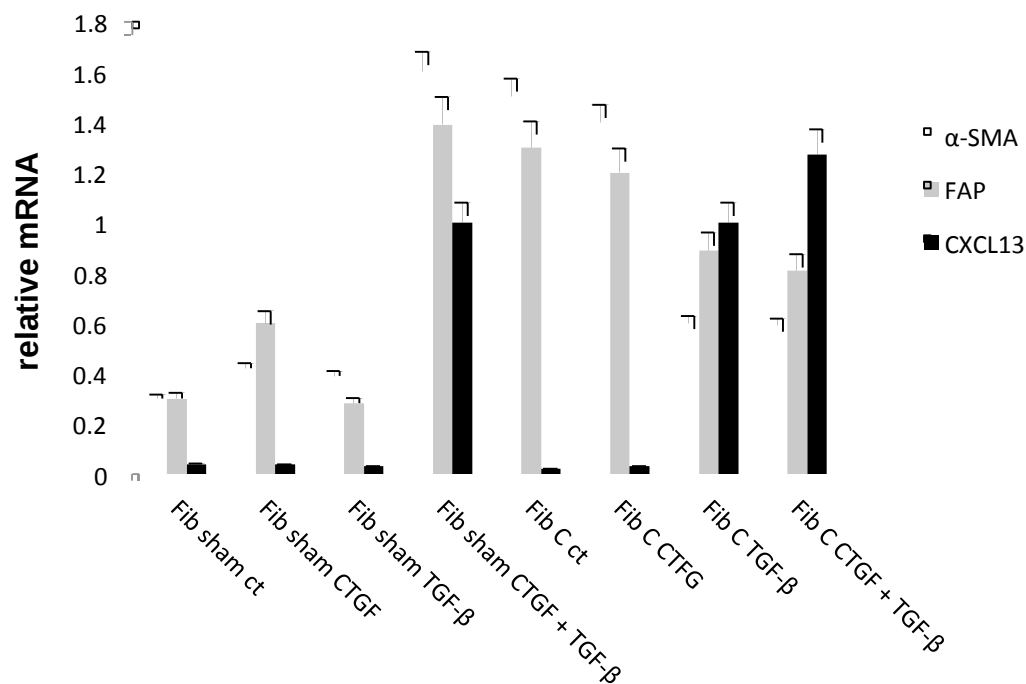


Figure 13

A



B

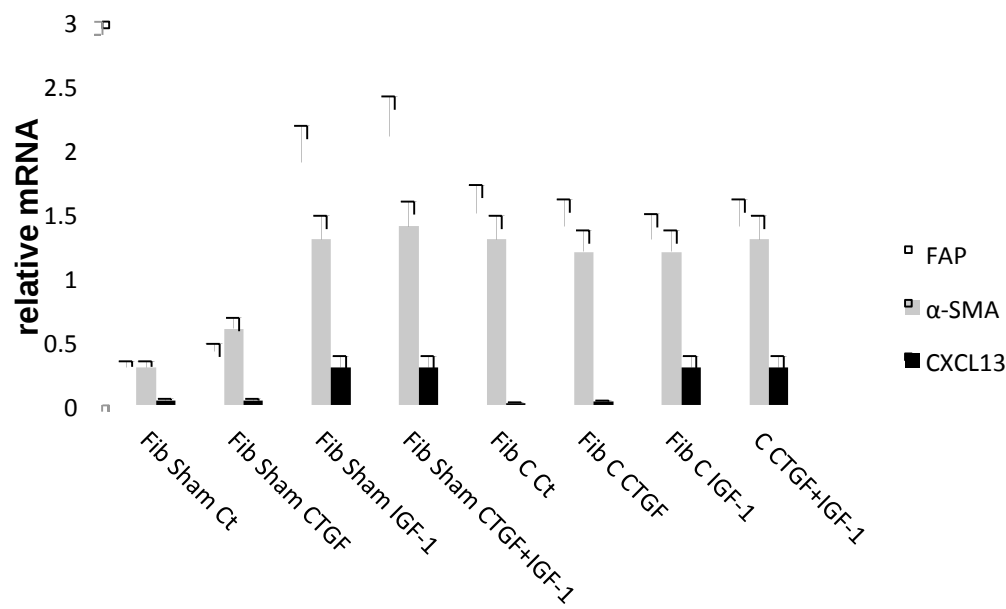


Figure 14

