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14. ABSTRACT Ovarian cancer remains a major health problem for women globally. While progress in developing new treatments strategies including immunotherapy for some types of cancer has been made, these approaches have had only limited impact in ovarian cancer. To this end, the current proposal was designed to test the central hypothesis that targeting the soluble RGDKGE collagen fragment may re-activate immune control of ovarian cancer and enhance the efficacy of immune checkpoint inhibitors by selectively disrupting a novel integrin-dependent signaling cascade. Our data suggest for the first time that ID8-VEGF and SKOV-3 tumors can generate the low molecular weight RGDKGE-containing collagen fragment that binds to the cell surface integrin alpha-V-beta-3. Cellular interactions with the RGDKGE-containing collagen fragment may lead to a unique signaling cascade that regulates YAP nuclear localization and may also control the expression of immune checkpoint molecules in selected immune cell lines. Selective targeting of the RGDKGE-containing collagen fragment inhibited ovarian tumor growth in murine models. Collectively, these observations provide new mechanistic insight into how an endogenously generated soluble collagen fragment may regulate ovarian tumor growth and may help with the development of alternative strategies to manage ovarian cancer.					
15. SUBJECT TERMS Tumor Associated Macrophages Lymphocyte Activation Gene Programmed Cell Death Ligand 1. Extracellular Matrix Immune Checkpoint Inhibitors Integrins Ovarian Cancer T-Cells Collagen Monoclonal Antibodies					
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1). Introduction:

Ovarian cancer remains a major health problem for women globally. While progress in developing new treatments strategies including immunotherapy for some types of cancer has been made, these approaches have had only minimal impact on ovarian cancer. Given these facts, discovering new molecular and cellular insight into the mechanisms that limit the efficacy of immunotherapy in ovarian cancer may lead to more effective treatment paradigms. In this regard, we made the observations that a highly conserved RGDKGE containing fragment of collagen that binds to the integrin receptor alpha-V beta-3, can be generated and secreted by a subset of M2-like macrophages. A soluble form of this RGDKGE collagen fragment potently induced angiogenesis and inflammation in vivo. An antibody (Mab XL313) that selectively binds the RGDKGE collagen fragment, blocked binding of this collagen fragment to integrin alpha-V beta-3 expressing cells and inhibited angiogenesis and inflammation in vivo. The current proposal was designed to test the central hypothesis that targeting the soluble RGDKGE collagen fragment may re-activate immune control of ovarian cancer and enhance the efficacy of immune checkpoint inhibitors by selectively disrupting a novel integrin-dependent signaling cascade.

2). Key Words:

- 1). Tumor Associated Macrophages
- 2). Yes-Associated Protein
- 3). Programmed Cell Death Ligand 1
- 4). Extracellular Matrix
- 5). LAG-3
- 6). Integrins
- 7). Ovarian Cancer
- 8). LATS1/2
- 9). Collagen
- 10). CTLA-4

3). Accomplishments:

Summary of major goals of project:

A). As outlined in aim-1, tasks 1 and 2, we proposed to test the effects of blocking cellular interactions with the soluble RGDKGE collagen fragment on ovarian tumor growth. In addition, we sought to determine whether this novel treatment strategy selectively enhances the therapeutic response of ovarian cancer to immune checkpoint inhibitors.

B). As outlined in aim-2, tasks 3 through 5, we proposed to determine how the soluble RGDKGE collagen fragment stimulates immunosuppression by activating a unique integrin signaling pathway in T-cells.

Summary of the overall accomplishments achieved for this project as they relate to the major goals described above. We have summarized below the results that have been completed for the studies proposed over the entire project period (April 2017 through April 2020). Due to significant and unexpected delays that occurred as a result of working with the original murine ID8 ovarian tumor model, a no-cost extension was granted for an additional 12-month period, which allowed us to make significant additional progress towards the overall goals of the project. In this final comprehensive scientific report, we provide a detailed summary of our current research accomplishments as they pertain to the tasks covered within the entire project as outlined in the statement of work.

Aim 1 Task 1. Analysis of the effects of Mab XL313 alone and in combination with immune checkpoint inhibitor.

Effects of Mab XL313 on subcutaneous ID8 tumor growth in vivo. To begin to examine the effects of targeting the RGDKGE collagen fragment on ovarian tumor growth in vivo, we first carried out a pilot experiment. Briefly, ID8 tumor cells were injected subcutaneously in mice and 5 days later following development of well-defined solid tumors, mice were treated with either non-specific control antibody or Mab XL313 specifically directed to the RGDKGE collagen fragment. As shown in figure 1, at the end of the 14-day treatment period, tumors from mice treated with Mab XL313 were approximately 35% reduced in size as compared to controls. However, due to outliers in this experiment, the reduction in size did not reach statistical significance. Surprisingly, observations from this initial experiment indicated that control treated mice showed evidence of regression from their initial size. These observations suggested that while Mab XL313 may have exhibited some anti-tumor activity, ID8 tumors appeared to exhibit spontaneous regression on their own irrespective of the treatment. These data suggest that the original subcutaneous ID8 ovarian tumor model examined may not represent an optimal model to study ovarian cancer in this setting.

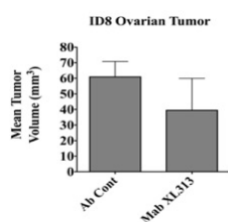


Figure 1. Effects of Mab XL313 on subcutaneous ID8 tumor growth in vivo. ID8 tumor cells (10×10^6 /mouse) were injected subcutaneously in C57BL/6 mice (N=8 per group). Beginning on day 5, mice were treated i.p. with non-specific control antibody (Ab Cont) or Mab XL313 (100ug 3x per week). Data bars indicate mean tumor volume $\pm$ S.E.M.

Analysis of immune cell infiltration of ID8 tumors over 14-day time course. Given our surprising preliminary observations suggesting that the ID8 tumors spontaneously regress irrespective of the treatment, we sought to assess whether this regression was associated with any time dependent changes in immune cell infiltration. Using similar experimental approaches, ID8 ovarian tumors were harvested on days-2 and 14 and analyzed. As shown in figure 2A, ID8 cells formed well-defined solid subcutaneous tumors but regressed over time without any treatment. Importantly, the lesions that did form were associated with stromal and immune cell infiltrates as indicated by H&E staining (Figure 2B). To further analyze these tumors, single cell suspensions were prepared and analyzed by flow cytometry for antigen markers including CD8, CD3, CD4, and CTLA-4. As shown in figure 2C and D, the relative levels of both CD3 and CD8 expressing T-cells increased with time from day-3 to day-14, while the levels of CD4 expressing cells appeared to peak on day-7 showing little if any increase from day-7 to day-14 (Figure 2E). Interestingly, the levels of CD4+/CTLA-4 double positive cells, which help mark immunosuppressive T-Reg-like cells significantly increased in a time dependent manner (Figure 2F).

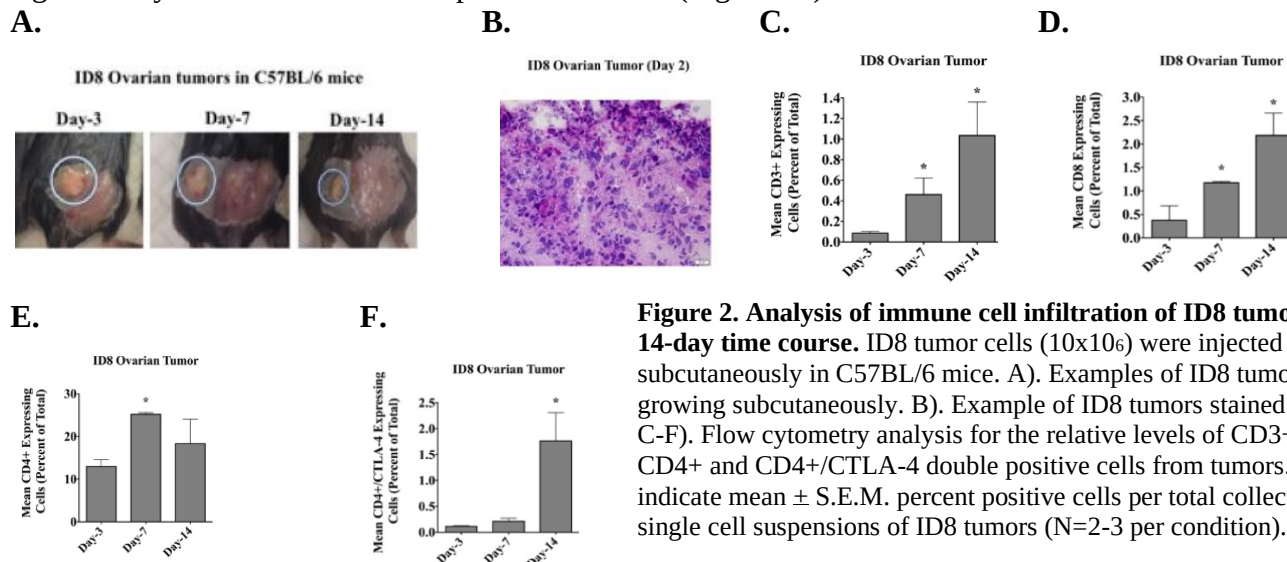


Figure 2. Analysis of immune cell infiltration of ID8 tumors over 14-day time course. ID8 tumor cells (10×10^6) were injected subcutaneously in C57BL/6 mice. A). Examples of ID8 tumors growing subcutaneously. B). Example of ID8 tumors stained by H&E. C-F). Flow cytometry analysis for the relative levels of CD3+, CD8+, CD4+ and CD4+/CTLA-4 double positive cells from tumors. Data bars indicate mean $\pm$ S.E.M. percent positive cells per total collected from single cell suspensions of ID8 tumors (N=2-3 per condition). *P<0.05

Analysis of stromal cell infiltration of ID8 tumors over 14-day time course. Next, we carried out experiments to examine the potential differential infiltration of additional stromal cells in ID8 tumors by flow cytometry using antibodies directed to LAG-3, PD-L1, Gr1, CD11b, F4/80 and PDGFR α . As shown in figure 3A, while a time dependent increase in the levels of LAG-3 expressing cells were observed by day-14, the number of PD-L1 expressing cells increased by day-7, then returned to lower levels similar to that observed on day-3 (Figure 3B). The relative levels of Gr1/CD11b double positive cells with characteristics of myeloid derived suppressor cells (MDSC) significantly decreased by day-14 as compared to day-3 (Figure 3C). Finally, while minimal changes were observed with F4/80 expressing macrophages (Figure 3D), a sharp increase in PDGFR α expressing cells were observed by day-14 (Figure 3E), however, this change did not reach statistical significance. These data are consistent with a time dependent differential accumulation of distinct subsets of stromal cells in ID8 tumors.

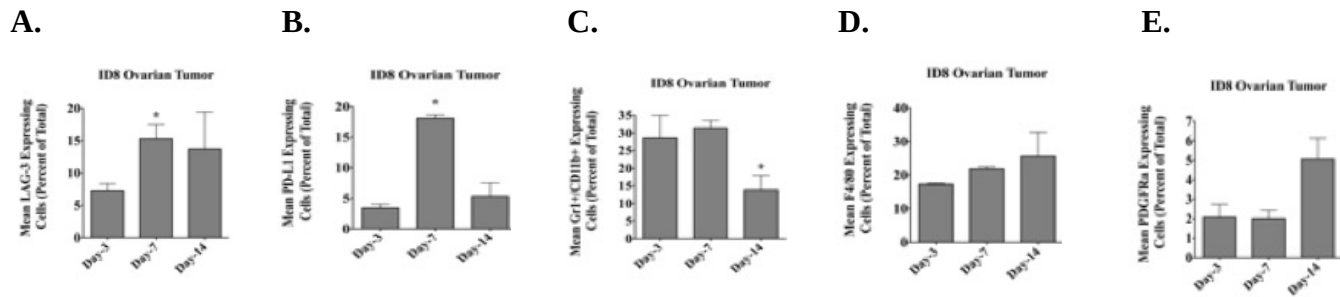


Figure 3. Analysis of stromal cell infiltration of ID8 tumors over 14-day time course. ID8 tumor cells (10×10^6) were injected subcutaneously in C57BL/6 mice (N=2-3 per group). Single cell suspensions were prepared from solid tumors and analyzed by flow cytometry for the indicated antigens. Data bars indicate the mean $\pm$ S.E.M percent positive cells per time point. *P<0.05.

Analysis of immune cell content of spleens from ID8 tumor bearing mice. Next, we sought to carry out similar experiments to examine potential differential immune cell composition of spleens from mice injected with ID8 tumor cells using flow cytometry and antibodies directed to CD8, CD3 CD4, CTLA-4, Gr1/CD11b, and LAG-3. Single cell suspensions were prepared from whole spleens and flow cytometry was performed. As shown in figure 4A-C, no significant changes were observed in the levels of CD8+, CD3+, or CD4+ positive T-cells in the spleens of ID8 tumor bearing mice between day-3 and day-14. Interestingly, a time dependent reduction was observed in the levels of CD4+/CTLA-4 double positive cells with characteristics of T-Reg-like cells in the spleens between days-3 and day-14 (Figure 4D). Moreover, the levels of Gr1/CD11b double positive cells with characteristics of MDSC, as well as LAG-3 expressing cells were reduced on day-7 as compared to either day-3 or day-14 (Figure 4-E and F).

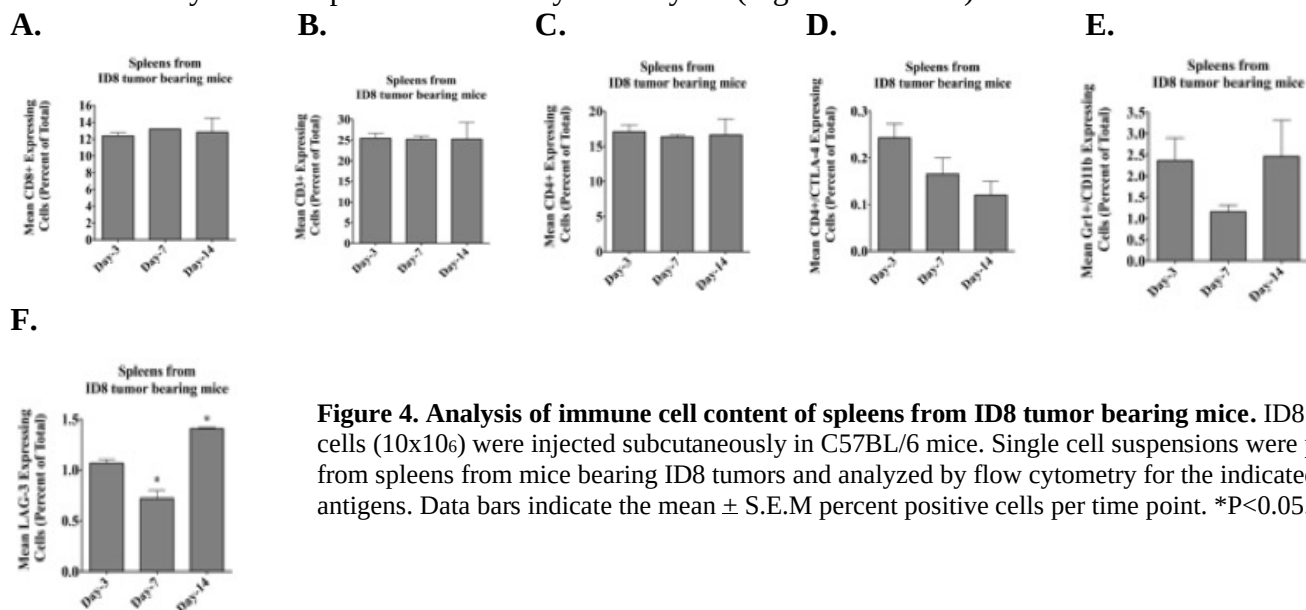


Figure 4. Analysis of immune cell content of spleens from ID8 tumor bearing mice. ID8 tumor cells (10×10^6) were injected subcutaneously in C57BL/6 mice. Single cell suspensions were prepared from spleens from mice bearing ID8 tumors and analyzed by flow cytometry for the indicated antigens. Data bars indicate the mean $\pm$ S.E.M percent positive cells per time point. *P<0.05.

Analysis of stromal cell content of spleens from ID8 tumors bearing mice. Next, we sought to perform similar experiments to examine the potential differential stromal cell composition of spleens from mice injected with ID8 tumor cells using flow cytometry and antibodies directed to PD-L1, F4/80 and PDGFR α . Single cell suspensions were prepared from whole spleens and flow cytometry was performed. As shown in figure 5A and B, no significant changes were observed in the levels of PD-L1 expressing cells or F4/80 expressing macrophages in the spleen from days-3 to day-14. While a small increase in the levels of PDGFR α expressing cells was observed in spleens of ID8 tumor bearing mice on day-14, this change did not meet statistical significance (Figure 5C).

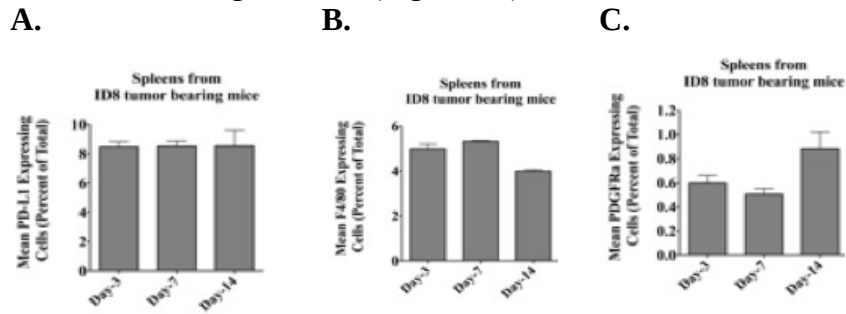


Figure 5. Analysis of stromal cell content of spleens from ID8 tumor bearing mice. ID8 tumor cells (10×10^6) were injected subcutaneously in C57BL/6 mice. Single cell suspensions were prepared from spleens from mice bearing ID8 tumors and analyzed by flow cytometry. Data bars indicate the mean $\pm$ S.E.M percent positive cells per time point. * $P < 0.05$.

Effects of Mab XL313 on intraperitoneal ID8 tumor growth in vivo. To examine the growth of intraperitoneal ID8 tumors in vivo, we injected ID8 tumor cells I.P in C57BL/6 mice and allowed them to grow for 4-weeks prior to treatment. After the 4-week growth period, mice were treated with either control antibody or Mab XL313 once per week for a total of 7 additional weeks. Mice were examined for the presence of peritoneal tumor lesions. As shown in figure 6A, multiple ID8 tumors were detected scattered throughout the peritoneal cavity and these tumors ranged from 1 to 4 foci per mouse and were from 2.0mg to 37mg. The ID8 tumors were found to be scattered throughout the peritoneal cavity of the majority (7 out of 8) control treated mice. In contrast only 5 out of 8 mice treated with Mab XL313 exhibited evidence of tumors and the foci that were detected were smaller. Quantification of the tumor burden (wet weights per mouse) indicated a significant inhibition of approximately 80% in the tumor burden in mice treated with Mab XL313 as compared to control (Figure 6B).

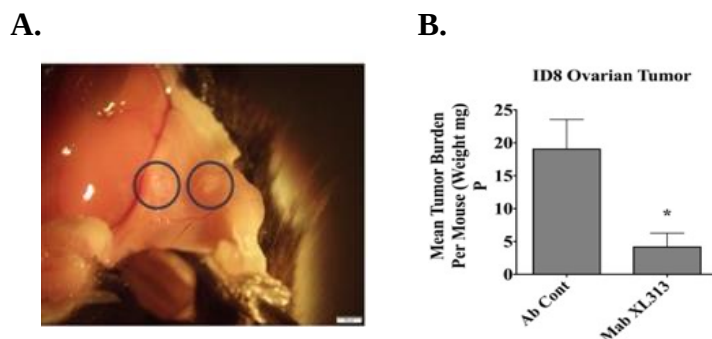


Figure 6. Effects of Mab XL313 on intraperitoneal ID8 tumor growth in vivo. ID8 tumor cells (10×10^6) were injected I.P in C57BL/6 mice ($N = 7-8$ per condition). Following a 4-week incubation period, mice were treated I.P. once per week for 7 additional weeks with 100ug of control antibody of Mab XL313. A). Example of I.P. tumor foci (circles). B). Quantification of total tumor burden per mouse. Data bars indicate mean ($\pm$ S.E.M) tumor weights from each condition. * $P < 0.05$.

Analysis of differential immune cell content of spleens from mice with intraperitoneal ID8 tumors treated with Mab XL313. Given our observations indicating differential immune cell content of the spleens from mice with subcutaneous ID8 tumors we carried out similar analysis by flow cytometry for the differential immune cell composition of the spleen from mice with intraperitoneal ID8 tumors. Briefly, single cell suspensions of whole spleens from the indicated mice were prepared and analyzed by flow cytometry using antibodies directed to immune cell antigens CD8, CD4 and LAG-3. As shown in figure 7A, no significant changes were observed in the levels of CD8 $^{+}$ T-cells in the spleens from each condition. Interestingly, a small but statistically significant reduction in CD4 $^{+}$ expressing cells were observed in the spleens from mice treated with Mab XL313 as compared to control antibody (Figure 7B). While a small

reduction in LAG-3 expressing cells was also observed in spleens from mice treated with Mab XL313, this did not reach statistical significance (Figure 7C).

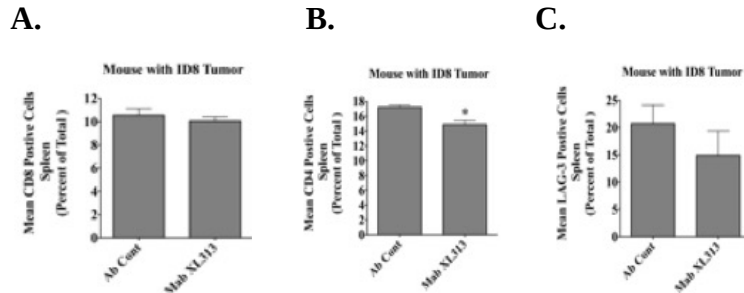


Figure 7. Analysis of differential immune cell content of spleens from mice with intraperitoneal ID8 tumors treated with Mab XL313. ID8 tumor cells (10×10^6) were injected i.p. in C57BL/6 mice and following 4 weeks of incubation, mice were treated with control antibody or Mab XL313 (100ug) 1x per week for an addition 7 weeks. Single cell suspensions were prepared of spleens from mice bearing ID8 tumors and analyzed by flow cytometry. Data bars indicate the mean $\pm$ S.E.M percent positive cells per time point. * $P < 0.05$.

Analysis of differential stromal cell content of spleens from mice with intraperitoneal ID8 tumors treated with Mab XL313. Next, we used a similar experimental approach using flow cytometry to examine any potential differential alteration in stromal cell composition of the spleen from mice bearing ID8 tumors. As shown in figure 8A, while a small reduction in the levels of F4/80 expressing macrophages were detected in the spleens from mice treated with Mab XL313, a statistically significant reduction in the overall level of PDGFR α expressing cells were observed in the spleens following treatment with Mab XL313 (Figure 8B).

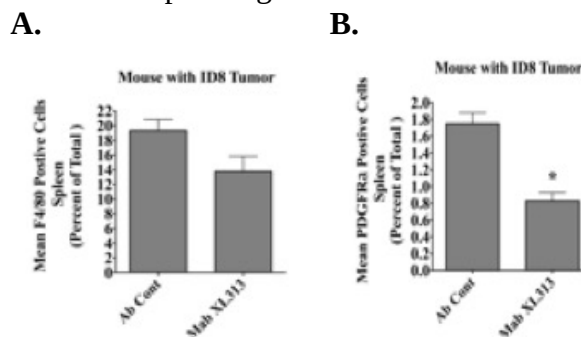


Figure 8. Analysis of differential stromal cell content of spleens from mice with intraperitoneal ID8 tumors treated with Mab XL313. ID8 tumor cells (10×10^6) were injected i.p. in C57BL/6 mice and following 4 weeks of incubation mice were treated (100ug) with control antibody or Mab XL313 1x per week for an addition 7 weeks. Single cell suspensions were prepared from spleens of mice bearing ID8 tumors and analyzed by flow cytometry. Data bars indicate the mean $\pm$ S.E.M percent positive cells per time point. * $P < 0.05$.

Analysis of differential stromal cell content of bone marrow from mice with intraperitoneal ID8 tumors treated with Mab XL313. Next, we used a similar experimental approach using flow cytometry to examine potential differential alterations in stromal cell composition of the bone marrow from mice bearing ID8 intraperitoneal tumors treated with Mab XL313 or control. As shown in figure 9A and B, a small but statistically significant reduction in the relative levels of F4/80 expressing macrophages and PDGFR α expressing cells were observed in the bone marrow of mice bearing ID8 intraperitoneal tumors treated with Mab XL313.

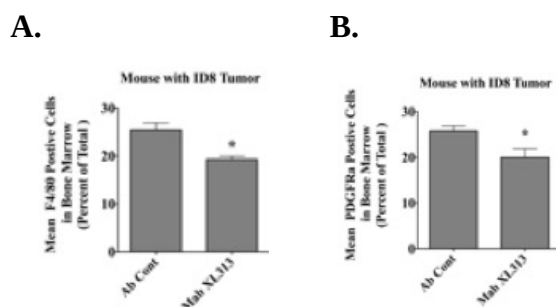


Figure 9. Analysis of differential stromal cell content from bone marrow from mice with intraperitoneal ID8 tumors treated with Mab XL313. ID8 tumor cells (10×10^6) were injected I.P in C57BL/6 mice and following 4 weeks of incubation, mice were treated (100ug) with control antibody or Mab XL313 1x per week for 7 weeks. Single cell suspensions were prepared from bone marrow of mice bearing ID8 tumors and analyzed by flow. Data bars indicate the mean $\pm$ S.E.M percent positive cells per time point. * $P < 0.05$.

Analysis of differential stromal cell content in peripheral blood from mice with intraperitoneal ID8 tumors treated with Mab XL313. We next used a similar experimental approach using flow cytometry to examine potential alteration in stromal cell composition of the peripheral blood from mice bearing ID8 intraperitoneal tumors treated with Mab XL313 or control. As shown in figure 10A, a statistically significant reduction in the relative levels of F4/80 expressing cells were detected within the blood of mice bearing ID8

intraperitoneal tumors treated with Mab XL313. However, no statistically significant change was observed in the levels of CD8⁺ cells in the blood from mice bearing ID8 tumors treated with Mab XL313.

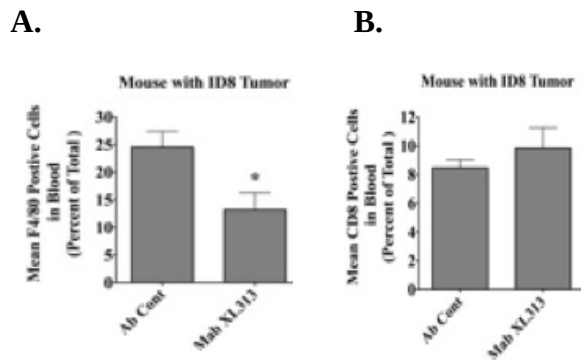


Figure 10. Analysis of differential stromal cell content of peripheral blood from mice with intraperitoneal ID8 tumors treated with Mab XL313. ID8 tumor cells (10×10^6) were injected I.P in C57BL/6 mice and following 4 weeks of incubation mice were treated (100ug) with control antibody or Mab XL313 1x per week for an addition 7 weeks. Single cell suspensions were prepared from blood from mice bearing ID8 tumors and analyzed by flow cytometry. Data bars indicate the mean $\pm$ S.E.M percent positive cells per time point. * $P < 0.05$.

Expression of PD-L1 within subcutaneous ID8 tumors growing in C57BL/6 mice. Given our experimental findings indicating differential levels of immune cells and other stromal cells in ID8 tumors and distinct changes in the levels of immune cells following treatment within Mab XL313, we examined the baseline distribution and co-localization of PD-L1 expressing cells in these ID8 tumors with different subsets of stromal cells including CD-31 expressing endothelial cells and alpha-SMA expressing stromal fibroblasts. As shown in figure 11A, PD-L1 (green) was widely distributed throughout these vascularized tumors, but showed little if any co-localization with CD-31 expressing endothelial cells (Red). In contrast, PD-L1 (Green) expression co-localized with alpha-SMA expressing stromal fibroblasts (Figure 11B). These data suggest that a large proportion of the PD-L1 was closely associated with alpha-SMA expressing stromal fibroblasts in these subcutaneous ID8 tumors.

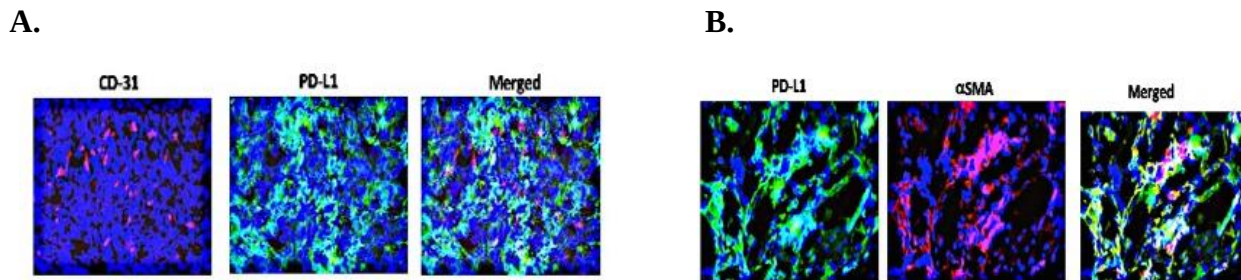
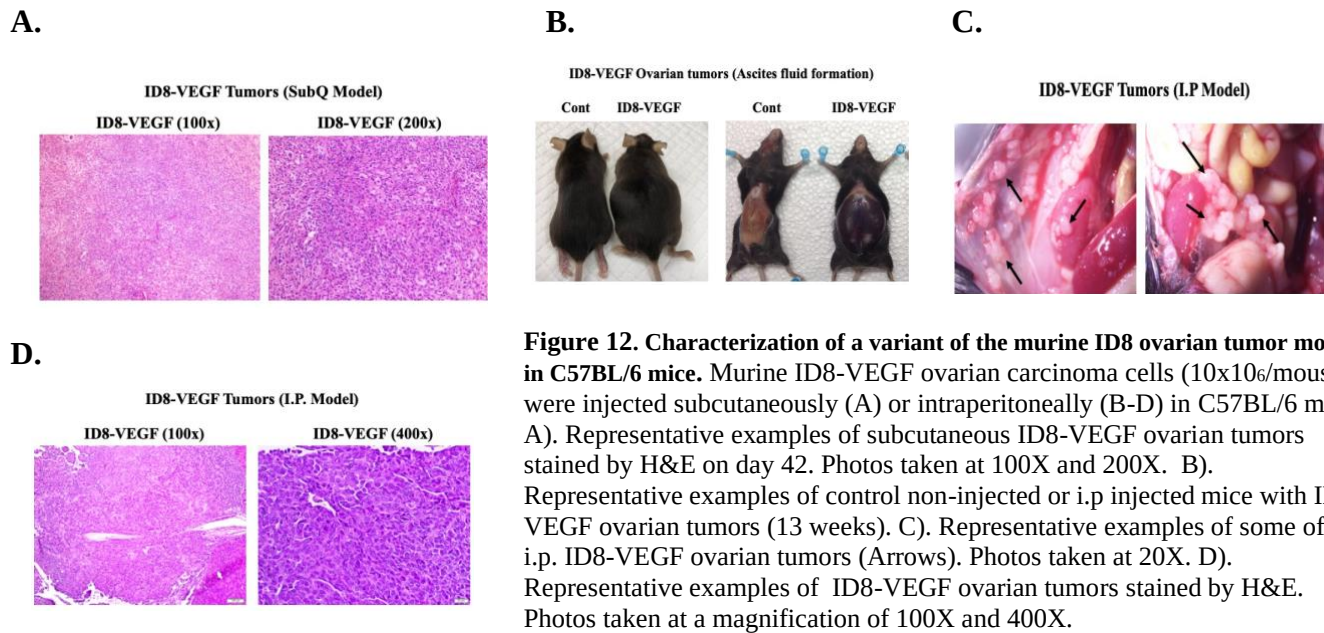


Figure 11. Expression of PD-L1 and alpha-SMA within subcutaneous ID8 tumors growing in C57BL/6 mice. ID8 tumor cells (10×10^6) were injected subcutaneously in C57BL/6 mice. ID8 tumors were dissected and frozen sections of tumors were co-stained with antibodies directed to PD-L1, CD-31 or alpha-smooth muscle actin (aSMA). A), Example of co-staining of ID8 tumors with PD-L1 (green) and CD-31 (Red). B), Example of co-staining of ID8 tumors with PD-L1 (Green) and aSMA (Red).

Establishment of ID8-VEGF ovarian tumor model. Given our surprising studies showing that ID8 tumor cells injected subcutaneously into C57BL/6 mice formed tumors, but their growth was slow and the tumors began to regress over time without treatment, we sought to establish a second ovarian tumor model by using ID8 tumor cells that overexpress the angiogenic growth factor VEGF (ID8-VEGF). As shown in figure 12A, injection of 10×10^6 ID8-VEGF cells subcutaneously into C57BL/6 mice formed well defined tumors. While these ID8-VEGF tumors also regressed, the kinetics of the regression was such that this model could be used to carryout additional experiments. In similar studies, ID8-VEGF cells were injected I.P. in order to establish an intraperitoneal ascites tumor model. Beginning at approximately 4 weeks after I.P injection of ID8-VEGF cells, mice started to exhibit signs of ascites fluid formation and by 13-weeks, mice clearly had formed ascites fluid as indicated by dramatically enlarged abdomens (Figure 12B). To confirm the formation of I.P tumors, mice were sacrificed and the abdominal cavity was dissected. As shown in figure 12C, numerous well-defined ID8-VEGF intraperitoneal tumors (black arrows) were observed throughout the abdominal cavity. These tumors were dissected and analyzed morphologically by H&E staining (Figure 12D).



Adaptive immune cells may contribute to early regression of ID8-VEGF tumors in vivo. Our studies have documented that early spontaneous regression of ID8 ovarian tumors growing in C57BL/6 mice were associated with differential infiltration of immune cells including CD4⁺ and CD8⁺ T-cell subsets. To examine whether the infiltration of these immune cells played a functional role in contributing to regression of ID8-VEGF tumors, we carried out an immune depletion study. Briefly, mice were pre-treated with non-specific control antibody or either anti-CD4 or anti-CD8 antibodies prior to injection with ID8-VEGF cells. To maintain immune depletion, mice were treated every 2 days with antibodies and tumor growth was monitored over 21-day time course. At the end of the treatment period mice were sacrificed and single cell suspensions of spleen tissues were examined by flow cytometry to confirm immune depletion. As shown in figure 13A and B, anti-CD4 and anti-CD8 antibodies significantly depleted their corresponding immune cell populations in these mice, while control antibody had no effect. Importantly, immune depleting CD8 positive T- cells slowed the regression of ID8-VEGF tumors by 21 days of growth, as the tumors from CD8 T-cell depleted mice were significantly larger than those tumors in controls depleted mice (Figure 13C). These data suggest that CD8⁺ T-cells play a functional role in the early regression of ID8-VEGF tumors in this model.

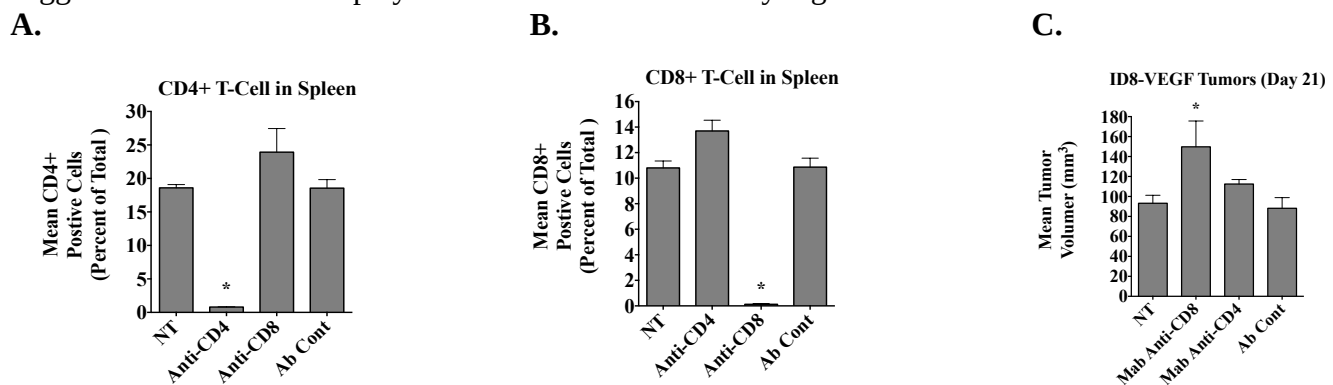


Figure 13. Adaptive immune cells contribute to early regression of ID8-VEGF ovarian tumors in vivo. C57BL/6 mice (N=4 per group) were pre-treated (i.p.) with anti-CD4, anti-CD8 or non-specific control antibody (Ab Cont) 24 hours prior to injection of tumor cells. Following injection of tumor cells, mice were further treated 3 x per week with the indicated antibodies for 21 days. Tumor size was monitored by caliper measurements. On day 21 mice were sacrificed and single cell suspensions prepared from spleens and the relative levels of CD4⁺ and CD8⁺ cells quantified by flow cytometry. A). Mean relative levels of CD4⁺ T-cells from immune depleted mice. B). Mean relative levels of CD8⁺ T-cells from immune depleted mice. C). Mean tumor size from immune depleted mice on day 21. Data bars indicate mean tumor size $\pm$ S.E.M.

Mab XL313 inhibits ID8-VEGF ovarian tumors in vivo. Given our studies, and the fact that we have now established the new ID8-VEGF ovarian tumor model, we sought to determine the effects of the anti-RGDKGE containing collagen fragment Mab XL313 on tumor growth in vivo. First, we sought to confirm that the ID8-VEGF tumor cells expressed the beta-3 integrin receptor that mediates interactions with the RGDKGE containing collagen fragment. As shown in figure 14A, beta-3 integrin could be readily detected in whole cell lysates of ID8-VEGF tumor cells as indicated by western blot. Next, we examined whether Mab XL313 directed to the RGDKGE collagen fragment could specifically block the ability of this collagen fragment to bind to ID8-VEGF tumor cells. ID8-VEGF cells were allowed to bind to the RGDKGE containing collagen fragment in the presence of either a non-specific control antibody or Mab XL313. As shown in figure 14B, Mab XL313 significantly inhibited the binding of the RGDKGE containing collagen fragment to ID8-VEGF cells by over 90% as compared to non-specific control antibody. To confirmed that the ID8-VEGF tumors growing in mice generate the RGDKGE-containing collagen fragment, we next examined the expression of the RGDKGE containing collagen fragment in ID8-VEGF tumors using Mab XL313. As shown in figure 14C, immunofluorescence staining analysis with Mab XL313 indicated expression of the RGDKGE containing collagen fragment (Red). Finally, we examined the effects of Mab XL313 on the growth of ID8-VEGF tumors in vivo. Briefly, mice were injected subcutaneously with ID8-VEGF (10x10⁶) cells and tumors were allowed to form for either 5 days (Figure 14D) or 14 days (Figure 14E) prior to treatment (arrows). Next, mice were treated with the indicated antibodies (100ug/injection) 2x per week. Treatment of mice with Mab XL313 significantly inhibited the growth of ID8-VEGF ovarian tumors by over 50% as compared to control antibody. Taken together, these studies indicate for the first time that selective targeting the RGDKGE containing collagen fragment can potentially inhibit ID8-VEGF tumor growth in vivo.

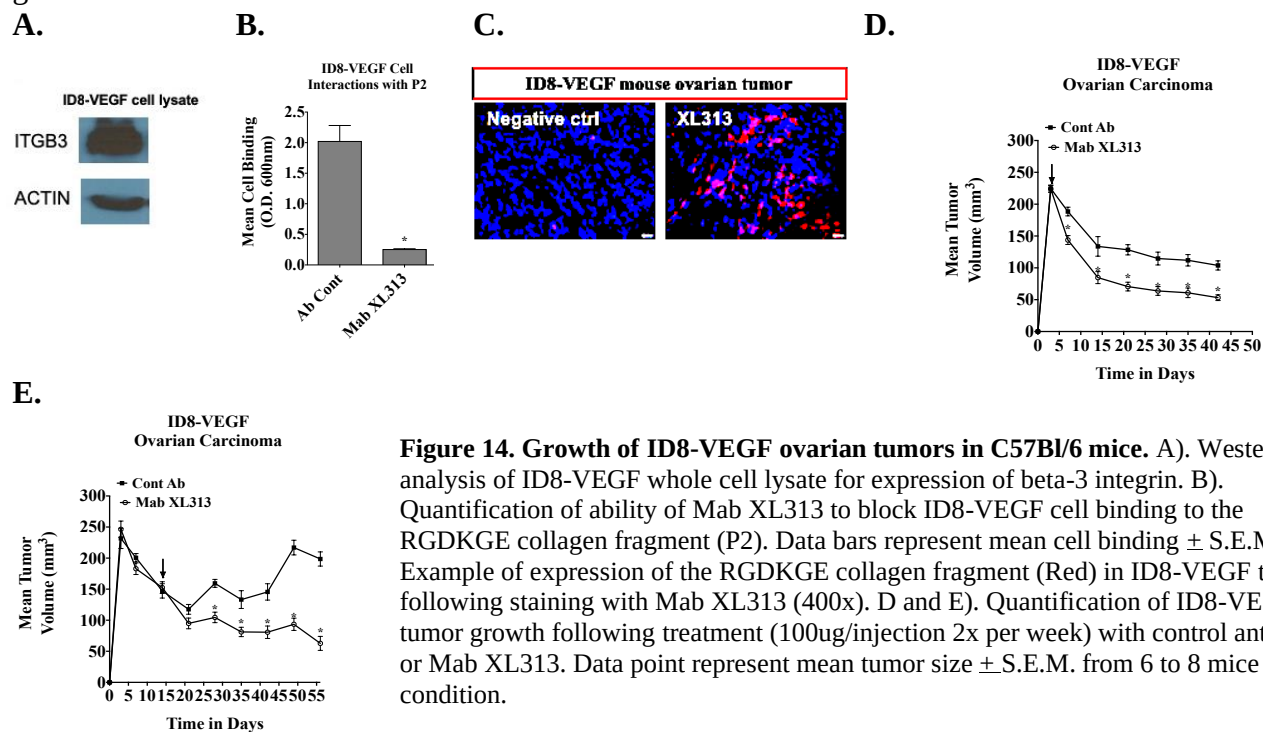
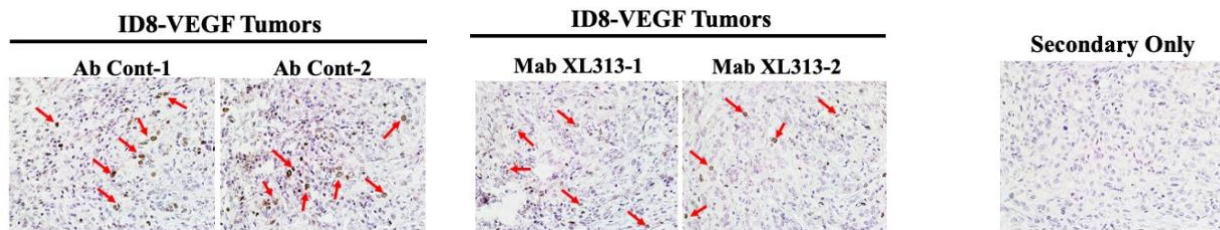


Figure 14. Growth of ID8-VEGF ovarian tumors in C57Bl/6 mice. A). Western blot analysis of ID8-VEGF whole cell lysate for expression of beta-3 integrin. B). Quantification of ability of Mab XL313 to block ID8-VEGF cell binding to the RGDKGE collagen fragment (P2). Data bars represent mean cell binding $\pm$ S.E.M. C). Example of expression of the RGDKGE collagen fragment (Red) in ID8-VEGF tumors following staining with Mab XL313 (400x). D and E). Quantification of ID8-VEGF tumor growth following treatment (100ug/injection 2x per week) with control antibody or Mab XL313. Data point represent mean tumor size $\pm$ S.E.M. from 6 to 8 mice per condition.

Alterations in proliferation and apoptosis in ID8-VEGF tumors in vivo. Next, we began to examine potential mechanisms by which Mab XL313 may inhibit tumor growth. In this regard, we first carried out a study to examine the expression of both proliferation (Ki67) and apoptosis (Caspase 3) markers in the ovarian tumors. Briefly, tumor sections from mice treated with control non-specific antibody or Mab XL313 were stained by immunohistochemistry for expression of Ki67 to help mark actively proliferating cells, and activated caspase 3 to help mark cells starting to undergo apoptosis. As shown in figure 15A, control treated ID8-VEGF treated tumors exhibited wide spread Ki67 staining (red arrows) indicating actively proliferating cells within these tumors. Interestingly, tumors from mice treated with Mab XL313 showed variable staining

for Ki67 with a trend toward reduced levels. In similar studies, tumor sections from each group were analyzed for expression of Caspase-3. As shown in figure 15B, tumors from mice treated with non-specific control antibody exhibited only a few scattered cells per field that stained positive for Caspase-3, consistent with relatively limited levels of apoptosis. While the relative levels of caspase-3 positive cells in tumors from mice treated with Mab XL313 were also limited and variable, a trend towards enhanced expression of caspase-3 was noted.

A.



B.

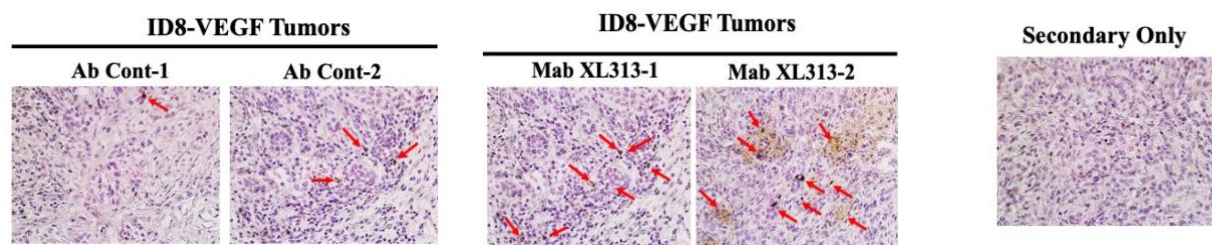


Figure 15. Detection of cellular proliferation and apoptosis in ID8-VEGF ovarian tumors in C57BL/6 mice.

Murine ID8-VEGF ovarian tumors from mice treated with control antibody or Mab XL313 (N= 3 per group) were analyzed by immunohistochemistry for expression of the proliferation marker (Ki67) or apoptosis marker (Caspase-3). A). Examples of ID8-VEGF tumors from mice treated with control antibody (left panel) or Mab XL313 (right panel) stained for proliferation marker Ki67. B). Examples of ID8-VEGF tumors from mice treated with control antibody (left panel) or Mab XL313 (right panel) stained for apoptosis marker (Caspase-3). Photos taken at a magnification of 200X.

Effects of Mab XL313 on intraperitoneal ID8-VEGF tumor growth and ascites formation in vivo. To examine the effects of Mab XL313 on the growth of intraperitoneal ID8-VEGF tumors and ascites fluid formation in vivo, we injected ID8-VEGF tumors cells I.P in C57BL/6 mice and allowed them to grow for 4 weeks prior to treatment. After the 4 week growth period, mice were treated with either control antibody or Mab XL313 2X per week for a total of a total of 13 weeks. Mice were examined for the presence of ascites fluid development as well as peritoneal tumor lesions growing within the diaphragms, a common site for colonization of ovarian tumors in both humans and mice. As shown in figure 16A, mice treated with control antibody readily formed ascites fluid as indicated by extensive distension of the peritoneal cavity, while mice treated with Mab XL313 showed dramatically reduced abdominal distension. Importantly, quantification of the mean ascites volume indicated that mice treated with Mab XL313 showed significantly reduced volume by 60% as compared to control antibody (Figure 16B). Moreover, ID8-VEGF tumor colonization of the diaphragms from mice treated with Mab XL313 was also observed (Figure 16C). Quantification of tumor colonization by wet weight of the diaphragms indicated a significant reduction in wet weight by approximately 30% as compared to control antibody treatment (Figure 16D). Taken together, these studies are in agreement with our previous studies and suggest that the RGDKGE collagen fragment plays a role in

regulating ovarian tumor growth in this model as specifically targeting the RGDKGE collagen fragment with Mab XL313 significantly inhibited the tumor growth and ascites formation *in vivo*.

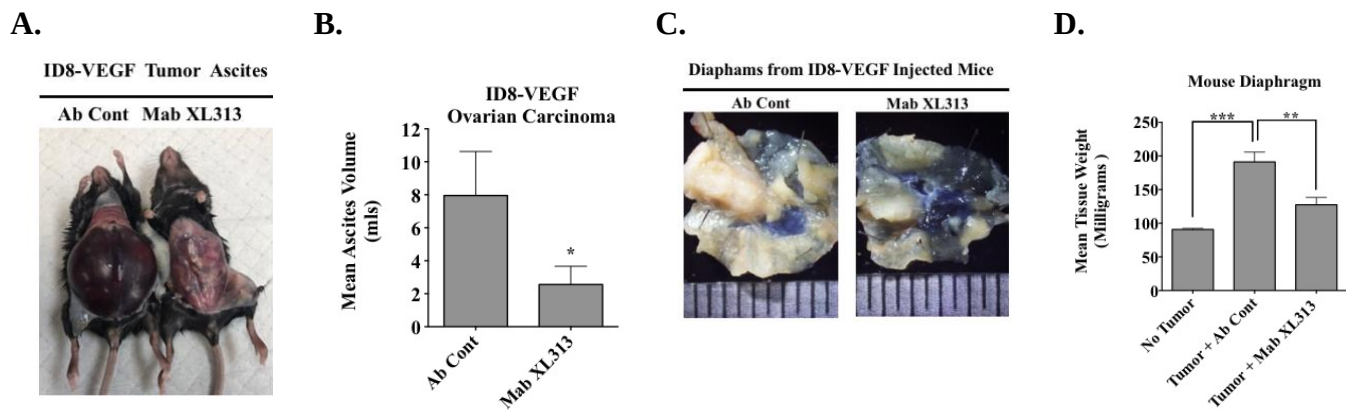


Figure 16. Effects of Mab XL313 on intraperitoneal ID8-VEGF tumor growth and ascites formation *in vivo*. ID8-VEGF cells (10x10⁶/mouse) were injected i.p. in C57BL/6 mice. Tumors were allowed to grow for 4-weeks. Mice were next treated 2x per week with non-specific control antibody or Mab XL313 (100ug/injection) for 13 weeks. A). Examples of abdominal distention between treatment groups. B). Quantification of the mean ascites fluid volume. Data bars indicate mean ascites volume $\pm$ S.E.M from 5 mice per group. C). Examples of ID8-VEGF tumor colonization within the mouse diaphragms. D). Quantification of the mean wet weights of the mouse diaphragms. Data bars indicate mean wet weights $\pm$ S.E.M from 5 mice per group. *P<0.05.

Effects of anti-CTLA-4 antibody on ID8-VEGF tumors *in vivo*. Given our studies suggesting that the murine immune system may impact ID8 tumor growth *in vivo*, we began to assess the effects of blocking the immune checkpoint molecule CTLA-4 on ID8-VEGF tumor growth. Briefly, we injected ID8-VEGF tumor cells i.p in C57BL/6 mice. Following a 4-week incubation period, mice were treated (100ug/mouse) 2x per week i.p with either non-specific control antibody or anti-CTLA-4 antibody. At the end of the 13-week treatment period, mice were sacrificed and intraperitoneal tumors dissected. As shown in figure 17A, as expected multiple scattered ID8-VEGF tumors were readily detected (N=5 to 6 per condition). Quantification of the mean total tumor burden (wet weight) per mouse showed no significant change between experimental conditions (Figure 17B). These data suggest that under these conditions, targeting CTLA-4 immune checkpoint failed to impact ID8-VEGF tumor growth. Given the technical issues encountered in establishing our ID8 ovarian tumor models studies examining the combination of Mab XL313 and anti-CTLA-4 were not completed.

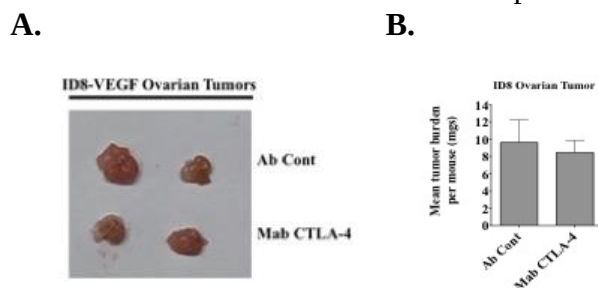


Figure 17. Effects of anti-CTLA-4 antibody on ID8-VEGF ovarian tumors *in vivo*. ID8-VEGF cells (10x10⁶/mouse) were injected i.p. in C57BL/6 mice. Tumors were allowed to grow for 4-weeks. Mice were next treated 2x per week with non-specific control antibody or Mab XL313 (100ug/injection) for 13 weeks. A). Examples of intraperitoneal ID8-VEGF tumors from each condition. B). Quantification of mean total tumor burden per experimental condition $\pm$ S.E.M.

Effects of anti-XL313 antibody on the levels of LAG-3 in bone marrow of mice with ID8-VEGF tumors. Our studies suggested that blocking immune cell interactions with the RGDKGE containing collagen fragment may alter the relative levels of the immune checkpoint molecule LAG-3. Therefore, we sought to examine the levels of LAG-3 expressing cells in the bone marrow of mice with ID8-VEGF tumors following treatment with anti-XL313 antibody directed to the RGDKGE collagen fragment. Briefly, mice were injected with ID8-VEGF tumor cells as described above and tumors were allowed to grow for 5 days. The mice were next treated 2x per week with either control non-specific antibody or Mab XL313 as

described above. At the end of the experiment (day-42) single cell suspensions were prepared from the bone marrow from the treated mice and analyzed for the relative levels of LAG-3 positive cells by flow cytometry. As shown in figure 18, significantly reduced levels of LAG-3 expressing cells were observed in the bone marrow of ID8-VEGF tumor bearing mice treated with Mab XL313 as compared to control antibody. These data are consistent with the possibility that blocking immune cell interactions with the RGDKGE collagen fragment may reduce the levels of LAG-3 in vivo.

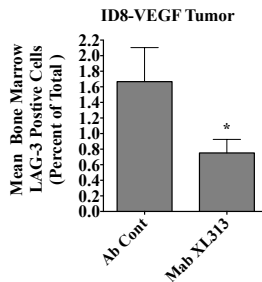


Figure 18. Effects of anti-XL313 Mab on the levels of LAG-3 in bone marrow of mice with ID8-VEGF tumors. Mice with ID8-VEGF ovarian tumors were treated (100ug/injection) 2x per week for 42 days with either non-specific control antibody (Ab Cont) or Mab XL313. Single cell suspensions of bone marrow cells isolated from each group was prepared and analyzed by flow cytometry. Data bars represent the mean relative percent of LAG-3 positive cells per group $\pm$ SEM.

Aim 1 Task 2. Analysis of the effects of Mab XL313 alone and in combination with immune checkpoint inhibitor in humanized mice.

Effects Mab XL313 on growth of SKOV-3 tumors in humanized mice. Briefly, NSG- humanized mice, in which the immune system of these mice partially reconstituted with human immune cells, were injected subcutaneously with SKOV-3 cells (3×10^6) and tumors were allowed to form for 3 days prior to treatment. Next, mice were treated 3x per week for 20 days. As shown in figures 19, treatment of mice with Mab XL313 significantly ($P < 0.05$) inhibited the growth of SKOV-3 ovarian tumors by approximately 40% as compared to control antibody. Taken together, these studies indicate that Mab XL313 not only inhibits murine ID8-VEGF tumors, but also potently inhibits human SKOV-3 tumors growing in mice with partially reconstituted human immune cells. Given the technical issues encountered in establishing our ovarian tumor models and the resulting delays, studies examining the combination of Mab XL313 and anti-CTLA-4 were not completed.

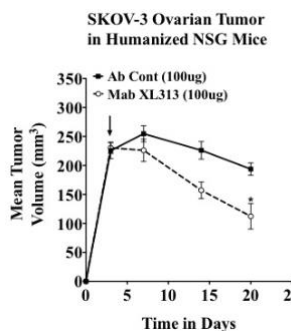


Figure 19. Effects of Mab XL313 on SKOV-3 tumor growth in humanized mice. SKOV-3 cells (3×10^6 /mouse) were injected subcutaneously in NSG-humanized mice. Tumors were allowed to grow for 3 days. Mice were next treated 3x per week with non-specific control antibody or Mab XL313 (100ug/injection) for 20 day. Quantification of mean tumor volume per experimental condition $\pm$ S.E.M. from 5 mice per condition

Analysis of T-cells of spleens, blood and tumors from mice with subcutaneous SKOV-3 tumors growing in humanized mice treated with Mab XL313. Given our observations indicating differential immune cell content of spleens from mice with subcutaneous ID8 tumors we carried out similar analysis by flow cytometry for CD4+ and CD8+ T-cells in the spleen, blood and tumors from humanized mice bearing SKOV-3 tumors. Briefly, single cell suspensions of whole spleens, tumors and peripheral blood from the indicated mice were prepared and analyzed by flow cytometry using antibodies directed to immune cell antigens CD3+/CD4+ and CD3+/CD8. As shown in figure 20A-C, the relative levels of CD3+/CD8+ double positive T-cells were increased following treatment with Mab XL313. However, do to the small sample size and some variation between animal, these data did not reach statistical significance. Interestingly, in similar studies, the relative levels of levels of CD3+/CD4+ double positive T-cells showed no change in the levels following treatment with Mab XL313 in the spleen or blood (Figure 20D and E), however, a trend toward an increase was observed in these CD3+/CD4+ T-cells within tumors from mice treated with Mab XL313

(Figure 20F). Taken together these data suggest an overall trend toward increased levels of human CD3+/CD8+ and CD3+/CD4 T-cells in tumors following treatment with Mab XL313 within the humanized mouse model, however, addition experiments will be needed to confirm these findings.

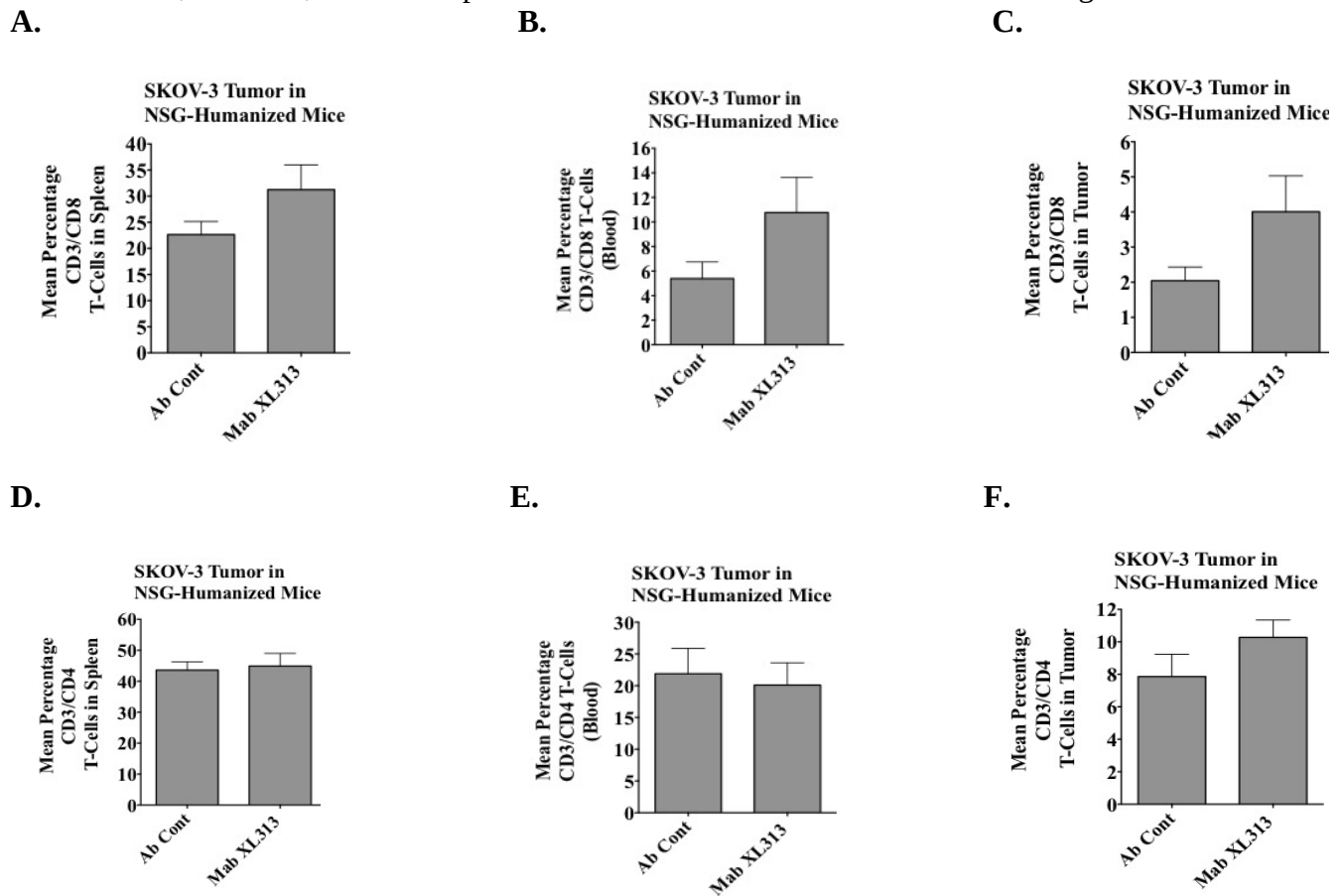


Figure 20. Analysis of T-cells of spleens, blood and tumors from mice with subcutaneous SKOV-3 tumors growing in humanized mice treated with Mab XL313. SKOV-3 tumor cells (3.0×10^6) were injected subcutaneously in NSG humanized mice and following 3-day of incubation, mice were treated with control antibody or Mab XL313 (100ug) 3x per week for 20 days. Single cell suspensions were prepared from, spleens, blood and tumors from the humanized mice bearing SKOV-3 tumors and analyzed by flow cytometry for CD3+/CD4 and CD3+/CD8 double positive T-cells. Data bars indicate the mean $\pm$ S.E.M percent positive cells per condition.

Aim-2 Tasks 3 and 4. Analysis of the roles of beta-3 integrin in facilitating the RGDKGE collagen fragment induced expression of PD-L1 and LAG-3.

Integrin beta-3 regulates expression of c-Myc in Jurkat T-cells. Our previous studies suggested that the RGDKGE collagen fragment recognized by Mab XL313 can bind to the integrin receptor alpha-v-beta-3. Given that our studies also suggested that the RGDKGE collagen fragment may regulate c-Myc, we sought to determine whether signaling through the beta-3 integrin might regulate c-Myc in T-cells. To examine this possibility, we first knocked down beta-3 integrin in Jurkat T-cells. As shown in figure 21A, transfection of anti-b3 specific shRNA efficiently reduced expression of beta-3 integrin as compared to non-targeting control shRNA (NT-Cont). As shown in figure 21B, knockdown of b3 integrin was associated with clear reduction of c-Myc. Taken together, these data provide evidence that beta-3 integrin may regulate c-Myc in these T-cells.

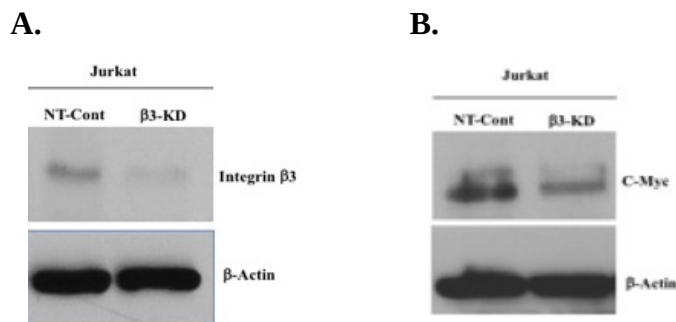


Figure 21. Integrin beta-3 regulates expression of c-Myc in Jurkat T-cells. Jurkat T-cells were transfected with either non-targeting control shRNA(NT-Cont) or beta-3 integrin specific shRNA (b3-Kd). Whole cell lysates were prepared and examined for expression of beta-3 integrin, c-Myc or actin as loading control. A). Western blot analysis of cell lysates for expression of beta-3 integrin or beta-actin. B). Western blot analysis of cell lysates for expression of c-Myc or beta-actin.

Beta-3 integrin regulates PD-L1 expression in Jurkat T-cells. Given our studies indicating a beta-3 integrin regulates expression of c-Myc in T-cells and c-Myc has been suggested to regulate expression of PD-L1 in some cell types, we examined whether beta-3 integrin may regulate PD-L1 in T-cells. As shown in figure 22, expression of PD-L1 was reduced in beta-3 knockdown Jurkat T-cells as compared control transfected cells. These data are consistent with a role for beta-3 integrin in regulating PD-L1 in this human T-cell line.

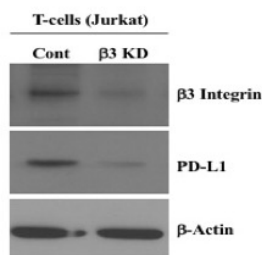


Figure 22. Integrin beta-3 regulates expression of PD-L1 in Jurkat T-cells. Jurkat T-cells were transfected with either non-targeting control shRNA(NT-Cont) or beta-3 integrin specific shRNA. Whole cell lysates were prepared and examined for expression of beta-3 integrin, PD-L1 or actin as loading control. Western blot analysis of cell lysates for expression of beta-3 integrin, PD-L1 or beta-actin.

Inhibition of c-Myc reduces expression of LAG-3 in Jurkat T-cells. Our current studies suggest that beta-3 integrin and c-Myc may play a central role in regulating the immune check point molecules PD-L1 and LAG-3 in T-cells. To conform a role for c-Myc in regulating LAG-3, we examined the effects of blocking c-Myc with a chemical inhibitor had on LAG-3 expression. As expected, inhibiting c-Myc readily reduced the expression of LAG-3 in Jurkat T-cells as compared to control (Figure 23).

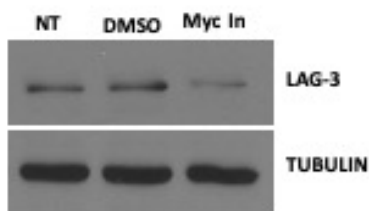
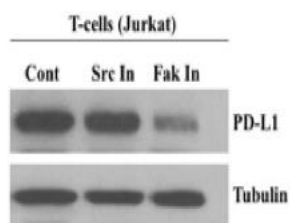


Figure 23. Inhibition of c-Myc reduces expression of LAG-3 in Jurkat T-cells. Jurkat T-cells were incubated with either control buffer DMSO or 100uM c-Myc inhibitor for 24 hours. Whole cell lysates were prepared and examined for expression of LAG-3 or tubulin as loading control. Western blot analysis of cell lysates for expression of LAG-3 or Tubulin.

Inhibition of Fak reduces expression of PD-L1 and LAG-3 in Jurkat T-cells. Our current studies suggest that beta-3 integrin may play a central role in regulating the immune check point molecules PD-L1 and LAG-3 in T-cells. Among the important down-stream effector molecules that are thought to facilitate signal transduction from integrin receptors include Src and FAK. To examine whether these effector kinases play a role in regulating the immune checkpoint molecules PD-L1 and LAG-3 in T-cells, we examined the effects of blocking these kinases had on immune checkpoint expression. Interestingly, while blocking FAK activity readily inhibited both PD-L1 and LAG-3 (Figure 24A and B), blocking Src failed to demonstrate any inhibitory activity on either PD-L1 or LAG-3 in Jurkat T-cells under these experimental conditions.

A.



B.

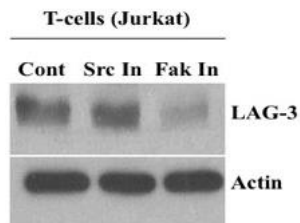
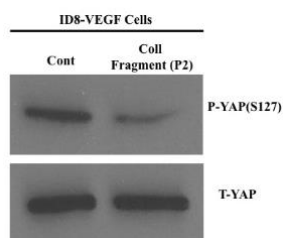


Figure 24. Inhibition of FAK reduces expression of PD-L1 and LAG-3 in Jurkat T-cells. Jurkat T-cells were incubated with control buffer DMSO or Src inhibitor (Src In) or Fak inhibitor (Fak In) for 24 hours. Whole cell lysates were prepared and examined for expression of PD-L1, LAG-3 or tubulin or actin as loading control. Western blot analysis of cell lysates for expression of PD-L1, LAG-3, or Tubulin and Actin.

Effects of ID8-VEGF cell binding to the RGDKGE collagen fragment P2 on YAP Activity. Previous studies suggest that both c-Myc and the Yes Associated Protein (YAP) may play a role in regulating PD-L1 expression. In this regard, our previous studies suggest that endothelial cell interactions with the RGDKGE collagen fragment regulate the localization and activity of YAP. To further study possible mechanisms by which the XL313 antibody may impact ID8-VEGF tumor growth and PD-L1 expression, we examined the effects of ID8-VEGF cell interaction with the RGDKGE collagen fragment P2 on YAP activity by examining the relative levels of phosphorylated YAP. The ability of YAP to accumulate in the nucleus can be controlled in part by a hippo-dependent phosphorylation event involving YAP phosphorylation on serine 127, which restricts nuclear YAP accumulation. In this regard, we examined YAP phosphorylation following ID8-VEGF cell binding to the RGDKGE collagen fragment P2. As shown in figure 25A, reduced levels of phosphorylated YAP were detected in ID8-VEGF cells binding to the RGDKGE collagen fragment as compared to control. Given these results, we next examined the relative levels of active nuclear YAP following ID8-VEGF binding to the RGDKGE collagen fragment P2 in the presence of Mab XL313 or control antibody. Consistent with the possibility that the RGDKGE collagen fragment P2 controls YAP activity, reduced levels of nuclear YAP were detected in ID8-VEGF cells following blocking binding the RGDKGE collagen fragment P2 with Mab XL313 (Figure 25B). Taken together, these data suggest for the first time, that direct interactions of ID8-VEGF cells with the RGDKGE collagen fragment P2 controls YAP activation. These data and other related findings are discussed in more detail within our manuscript (Han, W., et al.) submitted for publication that is attached in the appendix.

A.



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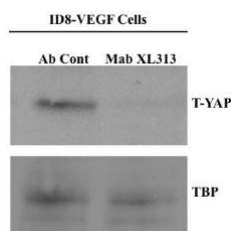


Figure 25. The RGDKGE containing collagen fragment P2 regulates YAP activity in ID8-VEGF cells. ID8-VEGF cells were allowed to bind the RGDKGE containing collagen fragment P2 for 1 hour in the presence of Mab XL313 or control non-specific antibody. Cytoplasmic and nuclear fractions were prepared. A). Western blot analysis of cytoplasmic fraction for phosphorylated YAP (P-YAP-S127) or total YAP (T-YAP). B). Western blot analysis of nuclear fraction for total YAP (T-YAP) or control TBP.

Effects of Mab XL313 on LATS1 phosphorylation in ID8-VEGF cells binding to the RGDKGE collagen fragment P2. Given our preliminary findings that suggest that ID8-VEGF cell binding to the RGDKGE collagen fragment P2 may regulate YAP phosphorylation, activation and nuclear accumulation, we sought to determine whether the ability of the RGDKGE collagen fragment to regulate YAP activity was associated with changes in hippo signaling given that the hippo effector kinase LATS1 is known to regulate phosphorylation of YAP on serine 127. To facilitate these studies we prepared whole cell lysates from ID8-VEGF cells attached to the RGDKGE collagen fragment P2 in the presence of Mab XL313 or control antibody and these lysates were analyzed by western blot for phosphorylated LATS1. As shown in figure 26, blocking ID8-VEGF cell binding to the RGDKGE collagen fragment P2 with Mab XL313 caused enhanced levels of phosphorylated LATS1 as compared to non-specific control antibody. These findings are consistent with the possibility that blocking the RGDKGE collagen fragment binding to ID8-VEGF cells may alter hippo signaling. These data and other related findings are discussed in more detail within our manuscript (Han, W., et al.) submitted for publication that is attached in the appendix.

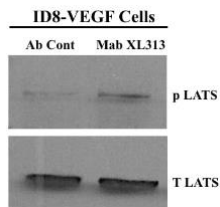


Figure 26. Effects of Mab XL313 on LATS1 phosphorylation in ID8-VEGF cells binding to the RGDKGE collagen fragment P2. ID8-VEGF cells were allowed to bind the RGDKGE containing collagen fragment P2 for 1 hour in the presence of Mab XL313 or control non-specific antibody and whole cell lysates were prepared. Western blot analysis for phosphorylated LATS1 or total LATS1.

Effects of cellular interactions with the RGDKGE collagen fragment on beta-3 integrin activation and c-Myc in ID8-VEGF tumor cells. Given our studies indicating that the RGDKGE collagen fragment binding to T-cells can regulate c-Myc and that c-Myc can regulate PD-L1, we sought to determine whether direct binding of the RGDKGE collagen fragment through the beta-3 integrin expressed on ID8-VEGF ovarian tumor cells could lead to activation and phosphorylation of beta-3 integrin. To examine this possibility ID8-VEGF cells were stimulated with the RGDKGE containing collagen fragment P2 or non-specific control peptide CP and the relative levels of phosphorylated and activated beta-3-integrin was assessed by western blot. As shown in figure 27A, stimulation of ID8-VEGF cells with the RGDKGE collagen peptide induced activation of beta-3-integrin as indicated by enhanced levels of phosphorylated (Tyr-747) beta-3-integrin. Importantly, blocking ID8-VEGF cell interactions with the RGDKGE collagen peptide using Mab XL313 inhibited beta-3 integrin phosphorylation (Figure 27B). Given these results, we sought to examine the effects of the RGDKGE collagen signaling through beta-3 integrin had on the relative levels of c-Myc. In this regard, similar studies were carried out and we examined the effects of the RGDKGE collagen fragment had on the relative levels of c-Myc mRNA. As shown in figure 27C and D, while stimulation of ID8-VEGF cells with the RGDKGE collagen fragment P2 enhanced c-Myc mRNA levels, selectively targeting the RGDKGE collagen fragment with Mab XL313 inhibited c-Myc levels as compared to control. Collectively, these studies provide evidence for the first time that the RGDKGE collagen fragment can not only regulate activation of beta-3 integrin and induced elevated levels of c-Myc in T-cells, but it also may regulate these molecules within the ovarian tumor cells themselves.

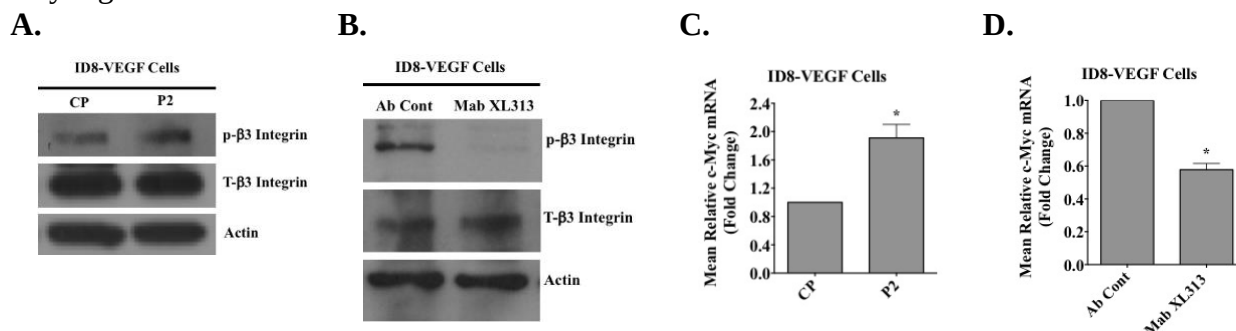
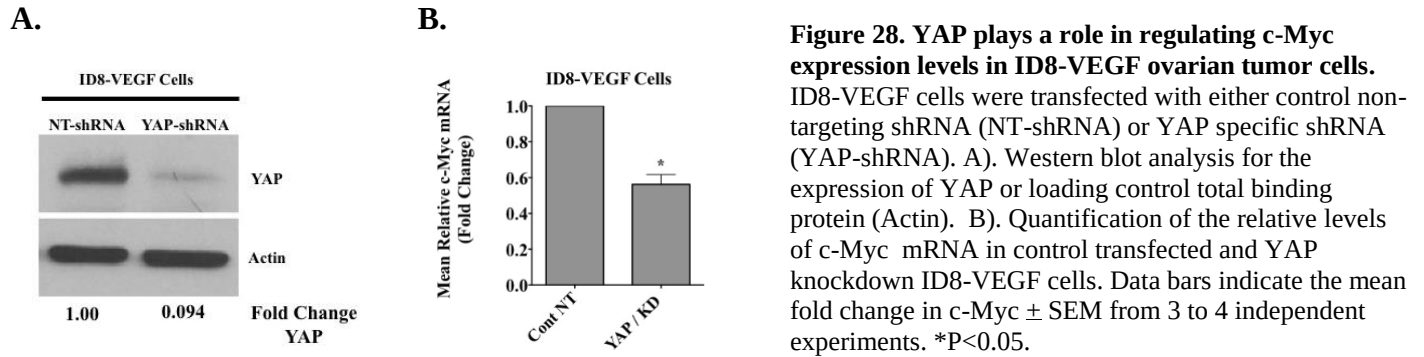


Figure 27. The RGDKGE collagen fragment activates beta-3 integrin and enhances c-Myc expression in ID8-VEGF tumor cells. ID8-VEGF cells were allowed to bind the RGDKGE containing collagen fragment (P2) or control peptide (CP) and whole cell lysates (A) or mRNA was prepared (B). A). Western blot analysis for the expression of total beta-3 integrin, phosphorylated (Tyr-747) beta-3 integrin, or loading control actin. B). Western blot analysis for the expression of total beta-3 integrin, phosphorylated (Tyr-747) beta-3 integrin, or loading control actin. C and D). Quantification of the relative levels of c-Myc mRNA. Data bars indicate the mean fold change in c-Myc $\pm$ SEM from 3 to 4 independent experiments. *P<0.05.

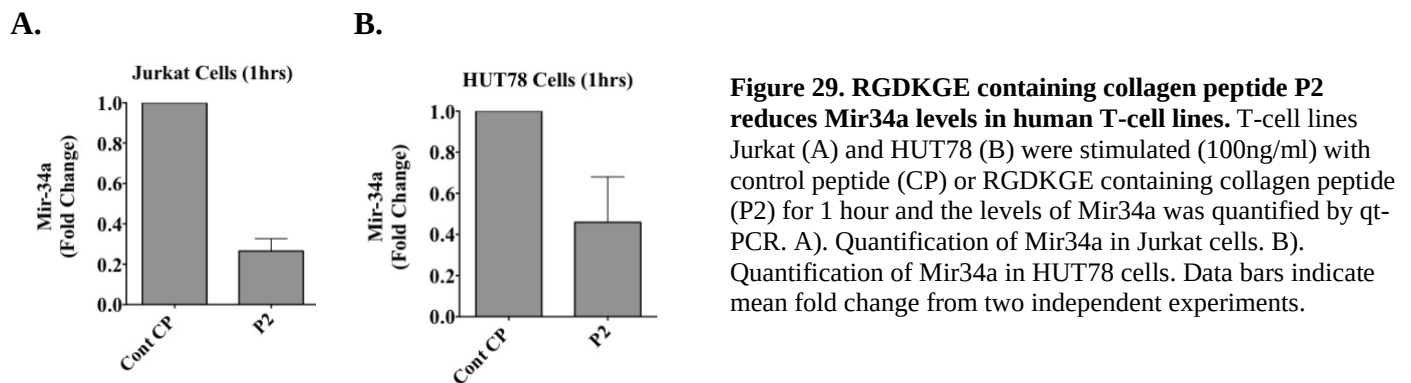
YAP plays a role in regulating c-Myc expression levels in ID8-VEGF ovarian tumor cells. Given our findings that c-Myc plays a role in regulating the expression of the immune checkpoint molecules PD-L1 and LAG-3 and that the RDGKGE collagen fragment signaling through beta-3 integrin can regulate the transcriptional co-activator Yes-Associated Protein (YAP), we sought to examine whether YAP may play a role in regulating c-Myc in ID8-VEGF ovarian tumor cells. To facilitate these studies we first knocked down expression of YAP using YAP specific shRNA. As shown in figure 28A, YAP expression was successfully reduced in ID8-VEGF (YAP-K/D) by shRNA as compared to transfection of non-targeting control construct (NT). Next, we examined the relative levels of c-Myc mRNA levels in these ID8-VEGF tumor cell variants. As shown in figure 28B, knocking down YAP resulted in significantly reduced levels of c-Myc as compared to control transfected cells. These data are consistent with a possibility that the ability of the RGDKGE

collagen fragment signaling through beta-3 integrin to control c-Myc might dependent in part on YAP. However, further studies will be needed to confirm this possibility.



Aim-2 Tasks 5 Analysis of the roles of mir34a in facilitating the RGDKGE collagen fragment induced expression of PD-L1 and LAG-3.

The RGDKGE containing collagen peptide P2 reduces Mir34a levels in human T-cell lines. Our previous studies suggest that the RGDKGE collagen fragment binding to β 3-integrin in Jurkat T-cells may enhance the relative levels of c-Myc as well as increase the levels of the immune check point molecule PD-L1. Given these results, and the fact that studies have implicated c-Myc in regulating Mir34a, we sought to determine whether the RGDKGE collagen peptide might play a role in regulating the relative levels of Mir34a. To facilitate these studies, we stimulated two independent human T-cell lines (Jurkat and HUT78) with the RGDKGE containing collagen peptide P2 and the relative changes in expression of Mir34a was quantified. As shown in figure 29A, stimulation of Jurkat T-cells with the RGDKGE containing collagen peptide P2 reduced the relative levels of Mir34a as compared to non-specific control peptide CP at 1hr post stimulation. Importantly, similar reduction was also observed following stimulation of HUT78 T-cells (figure 29B). These preliminary studies suggest for the first time that T-cell interaction with the RGDKGE containing collagen peptide P2 may regulate expression of Mir34a. Given our previous studies indicating that the RGDKGE collagen peptide can stimulate c-Myc, taken together these studies are consistent with the possibility that the RGDKGE collagen fragment stimulates signaling through beta-3 integrin resulting in enhanced nuclear accumulation of YAP which may activate c-Myc which in turn may enhance expression of Mir34a which may ultimately lead to reduction of PD-L1. However, further detailed studies will be needed to confirm this potential mechanism.



What opportunities for training and professional development has the project provided? Nothing to report.

How were the results disseminated to communities of interest? First, an abstract and poster detailing some of our early preliminary findings was submitted and accepted for presentation at the virtual 2020 AACR meeting (see attached in appendix). Second, a manuscript entitled “An RGDKGE containing cryptic collagen fragment regulates phosphorylation of LATS1 and controls ovarian tumor growth by a YAP- dependent mechanism” has been prepared and has been submitted for publication to the American Journal of Pathology (see attached in appendix).

4). Impact.

A). What was the impact on the development of the principal disciplines of the project? Our new data detailed in this report provides critical new immunological insight into why subcutaneously implanted ID8 ovarian tumors may regress over time. Specifically, our new data is consistent with an early infiltration of activated CD8+ T-cells that contribute to inhibiting tumor growth, as specifically depleting these T-cells resulted in slowing spontaneous regression and enhanced the overall size of these tumors as compared to control. These studies may assist in the optimization and use of the ID8 model to study cellular and molecular mechanisms of ovarian tumor biology. In addition, our exciting new data also suggest for the first time that ID8 and SKOV-3 tumor cells can generate the RGDKGE collagen fragment and importantly can directly bind this collagen fragment, which was shown for the first time to regulate YAP activity in these tumor cells. Moreover, our studies also provide evidence that ovarian tumor cell interaction with the RGDKGE containing collagen fragment can regulate LATS1, a critical effector kinase that within the hippo signaling cascade that regulates YAP activity and play a critical role in the anti-tumor activity of Mab XL313. These novel findings provide evidence for a possible mechanism by which Mab XL313 may inhibit tumor growth. Taken together, our exciting new data provides new mechanistic insight that may help optimize the use of Mab XL313 to treat ovarian cancers.

B). What was the impact on other Disciplines? Nothing to report.

C). What was the impact on technology transfer? Nothing to report.

D). What was the impact on society beyond science and technology? Nothing to report

5. Changes or Problems. During the current reporting period, we made the surprising observations that while murine ID8 ovarian tumor cells injected subcutaneously into C57BL/6 mice did form tumors, their growth was quite slow and these tumors began to regress over time without treatment. These unexpected findings resulted in significant delays in carrying out several of our planned experiments and thus, we were not able to complete the examination of the effects of the combination of Mab XL313 and anti-CTLA-4 on tumor growth in vivo. Given this delay we requested a no-cost extension from the DOD which was recently approved. These unexpected technical issues caused significant delays and made it difficult to carry out some of the tumor experiments using this murine cell line and significantly slowed our progress on additional experiments. In order to solve this problem, we began to establish an alternative ID8 cell line model with different growth characteristics. Specifically, we sought to establish a second ID8 ovarian tumor model by using murine ID8 tumor cells in which the cells overexpress the angiogenic growth factor VEGF (ID8-VEGF). In order to use this cell line in vivo we needed to amend our IACUC animal protocol. The amended protocol was approved by both the IACUC at Maine Medical Center as well as by the DOD. We now have established this alternative ID8-VEGF ovarian cancer model which allowed us to make significant additional progress toward the overall goals of our proposal.

6. Products: (Bibliography of all publications and meeting abstracts – Final report requirement)

A). Manuscripts:

A review article was published. Han, X., Caron, J. M., and Brooks, P. C. Cryptic collagen elements as signaling hubs in the regulation of tumor growth and metastasis. J. Cell. Physiol. DOI:10.1002/jcp.29752. (see attached in appendix).

An abstract and poster was submitted to the 2020 AACR annual meeting and was accepted as a poster presentation. (see attached in appendix).

A research article which is based in part, on experiments from this study has been submitted for publication entitled “An RGDKGE containing cryptic collagen fragment regulates phosphorylation of LATS1 and controls ovarian tumor growth by a YAP-dependent mechanism” (see attached appendix).

B). Websites of Other Internet sites: Nothing to report.

C). Inventions, patent applications, and/or licenses: Nothing to report.

D). Other Products: Nothing to report.

7). Participants and other collaborating organizations.

Name:	<i>Peter Brooks</i>			
Project Role:	<i>Principal Investigator</i>			
Researcher Identifier (e.g. ORCID ID):				
Project Year	Year 1	Year 2	Year 3	Total
Nearest person month worked:	1.8 CM	1.8 CM	1.8 CM	5.4 CM
Contribution to Project:	<i>Provides overall scientific direction, analysis, writes manuscripts, supervises Staff Scientist.</i>			
Funding Support:	<i>N/A</i>			

Name:	<i>Xianghua Han</i>			
Project Role:	<i>Staff Scientist</i>			
Researcher Identifier (e.g. ORCID ID):				
Project Year	Year 1	Year 2	Year 3	Total
Nearest person month worked:	6 CM	6 CM	7 CM	19 CM
Contribution to Project:	<i>Performs cell biological, molecular and animal experiments.</i>			
Funding Support:	<i>N/A</i>			

Has there been a change in the active other support of the PD/PI or senior Key personnel since the last reporting period? N/A – Final Report

What other organizations were involved as partners? None

Special Reporting requirements: N/A

Appendix:

1). Publication: Han, X., Caron, J. M., and Brooks, P. C. Cryptic collagen elements as signaling hubs in the regulation of tumor growth and metastasis. J. Cell Physiol. DOI: 10.1002/jcp.29752. 2019.

2). Abstract/Poster: The XL313Cryptic Collagen Epitope Regulates Ovarian Tumor Growth by a YAP Associated Mechanism. 2020 AACR annual meeting.

3). Research Manuscript Submitted: Han, X., Caron, J. M., Lary, C. W., Sathyanarayana P., Vary, C., and Brooks, P. C. An RGDKGE containing cryptic collagen fragment regulates phosphorylation of LATS1 and controls ovarian tumor growth by a YAP-dependent mechanism. Submitted to American Journal of Pathology. June 2020.

REVIEW ARTICLE

Cryptic collagen elements as signaling hubs in the regulation of tumor growth and metastasis

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Abstract

Structural remodeling of the extracellular matrix is a well-established process associated with tumor growth and metastasis. Tumor and stromal cells that compose the tumor mass function cooperatively to promote the malignant phenotype in part by physically interacting with intact and structurally altered matrix proteins. To this end, collagen represents the most abundant component of the extracellular matrix and is known to control the behavior of histologically distinct tumor types as well as a diversity of stromal cells. Although a significant molecular understanding has been established concerning how cellular interactions with intact collagen govern signaling pathways that control tumor progression, considerably less is known concerning how interactions with cryptic or hidden regions within remodeled collagen may selectively alter signaling cascades, or whether inhibition of these cryptic signaling pathways may represent clinically effective therapeutic strategies. Here, we review the emerging evidence concerning the possible mechanisms for the selective generation of cryptic or hidden elements within collagen and their potential cell surface receptors that may facilitate signal transduction. We discuss the concept that cellular communication links between cell surface receptors and these cryptic collagen elements may serve as functional signaling hubs that coordinate multiple signaling pathways operating within both tumor and stromal cells. Finally, we provide examples to help illustrate the possibility that direct targeting of these unique cryptic signaling hubs may lead to the development of more effective therapeutic strategies to control tumor growth and metastasis.

KEYWORDS

angiogenesis, collagen, integrins, metastasis, tumor growth

1 | INTRODUCTION

It is now well accepted that the growth and metastatic spread of tumors depends on a complex and interconnected set of processes governed by reciprocal communication links between stromal and tumor cells within the local tissue microenvironment (Figure 1). Among the most well-studied stromal cells that contribute to tumor growth and metastasis are endothelial cells, phenotypically diverse subsets of activated fibroblasts, and a variety of differentially polarized immune cells from the innate and adaptive immune systems

(Alkasalias, Moyano-Galceran, Arsenian-Henriksson, & Lehti, 2018; DeNardo & Ruffell, 2019; Rivera & Bergers, 2015). Due in part to the extensive cellular and molecular heterogeneity that exists within tumors and its associated stroma, it is not surprising that therapeutic strategies designed to target a single molecular pathway or a single cell type have met with only modest clinical activity.

Given the diversity of signaling cascades controlling tumor growth and metastasis, as well as the diverse stromal and tumor cell types within which these signaling cascades are operating, efforts are now underway to identify potential signaling hubs or discrete entities

Multiple cellular components that contribute to tumor growth and metastasis

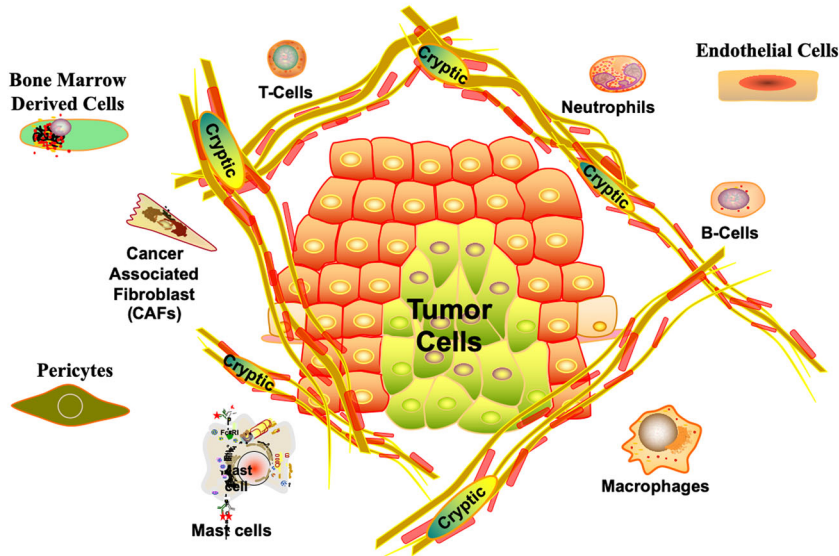


FIGURE 1 Complexity of the multiple components that compose the tumor microenvironment. The tumor microenvironment can be thought of as a complex interconnected compartment composed of phenotypically diverse cell types, secreted soluble factors, and an array of different extracellular matrix molecules. The tumor microenvironment is not static but rather dynamic with changes occurring in cellular constituents, molecular composition, biophysical characteristics, and structural integrity. Collectively, this complex and integrated compartment governs multiple characteristics of malignant tumor behavior

that coordinate multiple signaling pathways that contribute to the malignant phenotype (Figure 2). Thus, it is possible that a strategy of targeting signaling hubs may represent an effective method for treating malignant tumors. However, this therapeutic strategy would require a selective targeting approach to limit the potential for unintended consequences. One interesting example of a signaling hub can be illustrated by the formation of the physical contact points formed between cells and the extracellular matrix (ECM). Cell-ECM interactions are known to influence the behavior of distinct stromal cells as well as histologically diverse tumor cell types

(Caron et al., 2016; J. Xu et al., 2001). These specific cell-ECM contact points facilitate the ability of cells to selectively sense and then coordinately translate an array of mechanical and biochemical cues from the ECM into multiple intracellular signaling pathways that help fine-tune cellular behavior within distinct tissue microenvironments. These cell-ECM communication links control either directly or indirectly, the vast majority of cellular processes critical for governing tumor growth and metastasis, ranging from modulating gene expression, cell adhesion, migration, invasion, proliferation and differentiation to altering cell survival and

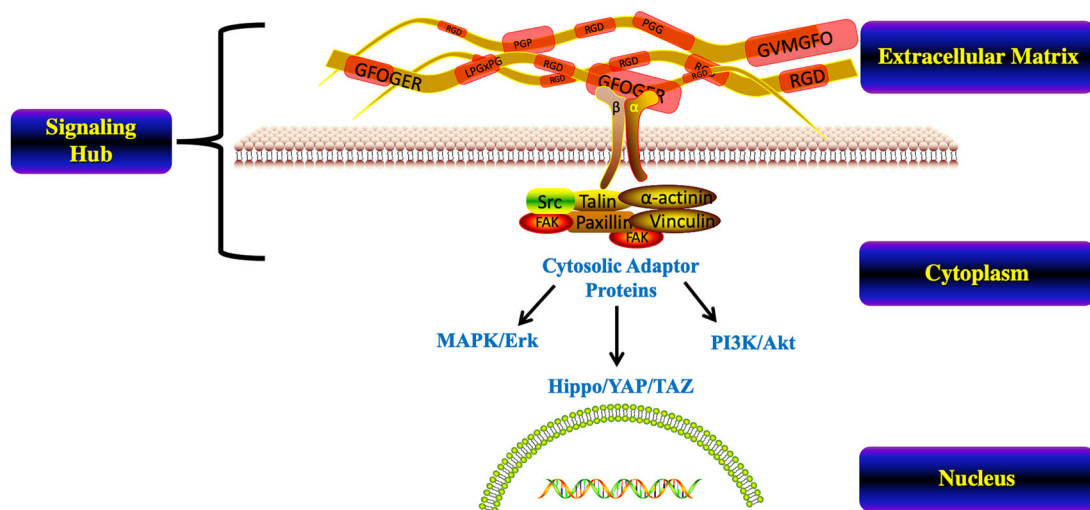


FIGURE 2 Functional signaling hubs coordinating information flow from outside to inside the cell. Schematic representation of a signaling hub, which can be thought of as a physical contact site between the cell and the external microenvironment that allows establishment of physical communication links between cell surface receptors and cytoplasmic adaptor molecules that facilitate initiation of multiple signaling cascades that control cell behavior. These signaling hubs can operate as relays that coordinate information transfer between the local tissue microenvironment and both tumor and stromal cells

apoptosis (Humphries, Chastney, Askari, & Humphries, 2019; Kechagia, Ivaska, & Roca-Cusachs, 2019).

From a general perspective, the ECM can be categorized into two major compartments including the basement membrane and interstitial matrix (Hynes & Naba, 2012; Mouw, Ou, & Weaver, 2014). These interconnected ECM compartments are composed of highly complex arrays of molecules and thus it would be well beyond the scope of this review to discuss in detail the diversity of components, structural features and functions of these tissue compartments. However, the most abundant of the structural ECM proteins that help compose both the basement membrane and interstitial matrix is the collagen family of molecules (Fidler, Boudko, Rokas, & Hudson, 2018; Ricard-Blum, 2011). Here, we have chosen to focus our discussion on how cellular interactions with collagen or more specifically, with structurally altered forms of collagen may be thought of as cryptic signaling hubs that might be selectively targeted to control tumor growth and metastasis.

2 | GENERAL COLLAGEN BIOSYNTHESIS, STRUCTURE, AND DIVERSITY

Early work from many investigators on the collagen family of ECM molecules helped define a diverse group of proline-rich proteins characterized by a tripeptide repeating structural unit (G-X-Y) with the amino acid glycine (G) often present at the third position in the triplet. The next two positions (X and Y) within the triplet unit are often occupied by proline or hydroxylated proline. This unique amino acid organization helps facilitate the appropriate folding of the individual collagen chains into its characteristic triple-helical structure (Kadler, 2017; Kadler, Hill, & Canty-Laird, 2008). A defining feature among all the collagen molecules is that they are generally composed of three individual chains, and these chains can be derived from different gene products. These individual chains exhibit a staggered orientation with respect to each other, which in turn, are folded into a supercoiled triple-helical conformation around a central axis (Canty & Kadler, 2005; Kadler, 2017; Kadler et al., 2008). These triple-helical structures can be composed of either homo-trimers of identical chains or in other cases, hetero-trimers composed of distinct groupings of genetically different chains giving rise to a diversity of molecules with distinct biochemical and biophysical properties (Canty & Kadler, 2005; Fidler et al., 2018; Kadler, 2017; Kadler et al., 2008; Ricard-Blum, 2011). These triple-helical molecules are further organized into higher-order structures such as fibrils, fibers, and interacting networks that form associations with other ECM molecules that help compose the interwoven structure of the ECM.

The biosynthesis, secretion, and organization of collagen into higher-order structures involves a complex and interconnected set of processes and will only be briefly discussed below. As with many ECM molecules, a variety of different growth factors and cytokines contribute to the regulation of collagen expression and structural organization. Among some of the more well-studied factors known to

regulate collagen expression are transforming growth factor- β (TGF β), interferon γ (INF γ), and interleukin-4 (IL-4) (Aoudjehane et al., 2008; Ghosh, Yuan, Mori, Chen, & Varga, 2001). Interestingly, some growth factors such as TGF β can be localized within the ECM in a latent form. Evidence suggests that a subgroup of integrin receptor that has the capacity to bind the arginine-glycine-aspartic acid (RGD) containing sequence within the inactive TGF β complex can lead to mechanical changes resulting in activation, thereby helping to facilitate localized TGF β activity (Khan & Marshall, 2016). Other cytokines, such as INF γ can bind to its receptor leading to the activation of a Stat-1 dependent signaling cascade, which ultimately leads to suppression of collagen expression (Ghosh et al., 2001). Conversely, TGF β signaling is thought to stimulate expression of collagen through a Smad-dependent signaling pathway (Ghosh et al., 2001). Interestingly, cytokine signaling not only influences the expression of collagen, but may also regulate collagen fibril assembly and structural organization. In this regard, recent studies suggest that among the proteins upregulated in a breast cancer cell line by TGF β are WSIP1/CCN4, a protein that was shown to bind to type-1 collagen and promote fiber linearization and enhanced tumor cell metastasis (Jia et al., 2019). Moreover, while IL-4 has been shown to enhance expression of collagen (Aoudjehane et al., 2008), signaling through the IL-4 receptor (IL-4R α) may also regulate collagen fibril assembly during skin repair (Knipper et al., 2015). Importantly, while cytokine signaling cascades not only regulate collagen expression and organization, other ECM molecules such as fibronectin, which has binding sites for collagen, can also regulate collagen fibril assembly and stability (Sottile & Hocking, 2002).

Although the overall biosynthesis, secretion, and fibrillogenesis of collagen can differ depending on the specific type of collagen, in general, collagen mRNAs are translated into pre-procollagen molecules and move through the ER and Golgi. Given the large size of these secreted molecules, unique mechanisms are thought to be used to transfer these large bulky proteins through the secretory compartments of the ER and Golgi including the heat shock protein-47 (HSP47) and the transport and Golgi organizing protein-1/melanoma inhibitory activity protein-3 (Malhotra & Erlmann, 2011; Ono, Miyazaki, Ishida, Uehata, & Nagata, 2012; Yuan, Kenny, Hemmati, Xu, & Schekman, 2018). Collagen can undergo extensive posttranslational modifications that include proline and lysine hydroxylation, glycosylation and disulfide crosslinking. Individual α -chains can interact through a complex selection process that results in different α -chain configurations that involve the C-terminal pro-peptide regions that facilitate the appropriate alignment to allow triple-helical folding to proceed from the C-terminus to the N-terminus of the molecule (Canty & Kadler, 2005; Fidler et al., 2018; Kadler, 2017; Kadler et al., 2008; Malhotra & Erlmann, 2011; Ono et al., 2012; Ricard-Blum, 2011; Yuan et al., 2018). These molecular events occur in the presence of specific enzymes and chaperone molecules such as PPI (peptidyl-prolyl cis-trans-isomerase) and HSP47. The procollagen molecules are packaged in vesicle coat protein coatomer COPII-coated vesicles needed for transport. Collagen molecules are eventually secreted out of the cell for further

processing that is thought to occur near specialized in-folded regions of the plasma membrane. Procollagen molecules are further processed by a combination of both C and N-terminal pro-peptidases, which help facilitate fiber bundling into larger higher-order networks (Canty & Kadler, 2005; Kadler et al., 2008). Depending on the particular collagen type, structural organization, and functional requirements for the specific tissue microenvironment where the collagen is deposited, the extent and specificity of the various posttranslational modifications such as hydroxylation and cross-linking help define both the mechanical and biochemical properties of the collagen. At least 28 distinct collagen types have been identified which can be organized based on their overall amino acid sequence, structure and Kadler et al., 2008-molecular configurations. Some of the major categories include fiber-forming collagens (type-I, -II, -III, -V, -XI), fiber-associated collagens with interrupted triple helices, basement membrane type collagen (type-IV) and/or network forming collagens (Fidler et al., 2018; Kadler, 2017; Ricard-Blum, 2011). Transmembrane collagens have also been identified (Fidler et al., 2018; Kadler, 2017; Ricard-Blum, 2011).

3 | CELLULAR BINDING TO COLLAGEN

Among the most well-studied functions of collagen is its pivotal role in providing mechanical stability and structural support in connective tissues. However, beyond the ability of collagen to function as simply a mechanical support, it is well known to mediate a wide array of signaling functions that impact nearly all aspects of cellular behavior. Critical to the ability of collagen to mediate signaling is its ability to bind to multiple cell surface receptors capable of transferring these collagenous biochemical and mechanical cues into the cell's interior. Given the abundance of the collagen family of proteins, their broad tissue distribution and their diverse functions, it is not surprising that cells are equipped with a wide range of receptors capable of binding to different forms of collagen. For example, some of the most well-studied collagen receptors include a series of molecules called integrins. Integrins are a family of transmembrane heterodimers composed of an α chain and a β chain derived from distinct gene products (Cooper & Giancotti, 2019; Humphries et al., 2019). These multifunctional receptors are capable of facilitating the ability of cells to sense and transfer both biochemical and mechanical information from the outside of the cell to the inside. Although integrins do not possess catalytic domains with enzymatic activity typical of more traditional signaling receptors, they can transmit signals by virtue of their complex protein-protein interactions occurring between their cytoplasmic tails and a variety of adaptor proteins, cytoskeletal molecules, and protein kinases, which collectively help form specialized signaling relays that have been categorized into multiple subtypes including nascent adhesions, focal adhesions and fibrillar adhesions (Geiger & Yamada, 2011). These critical adhesive structures provide a series of molecular linkages between the ECM, transmembrane integrin receptors, adaptor proteins and cytoskeletal elements, thereby coordinating the transfer of the vast amount of biochemical and mechanical information present within different extracellular environments.

Integrin binding can initiate unique conformational changes within the individual integrin chains, resulting in physical separation of the cytoplasmic tails of the α and β subunits (Cooper & Giancotti, 2019; Geiger & Yamada, 2011; Humphries et al., 2019). These mechanical alterations in structure ultimately facilitate recruitment and assembly of distinct combinations of accessory molecules that in turn, promote precise activation of many different downstream signaling cascades, some of which include Rho/Rac, MAP/Erk, PI3K/Akt, and Hippo-YAP/TAZ pathways to name just a few (Cooper & Giancotti, 2019; Geiger & Yamada, 2011; Humphries et al., 2019). Among the best-studied integrins capable of binding to triple-helical collagen are integrins $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$, and $\alpha11\beta1$, and these integrins can bind to intact triple-helical collagen through regions containing the amino acid sequences GFOGER where O indicates hydroxyprolines (Hamaia & Farndale, 2014; Popova, Lundgren-Akerlund, Wiig, & Gullberg, 2007; Zeltz & Gullberg, 2016). Although other collagen sequences have also been shown to bind to integrins, the ability of integrins to interact with collagen is not simply dependent on primary amino acid sequence. For example, posttranslational modifications of collagen and structural changes in the precise geometry and orientation of the contact residues help guide the ability of integrins to bind these ECM proteins (Hamaia & Farndale, 2014; Popova et al., 2007; Zeltz & Gullberg, 2016). Structural changes of collagen mediated by mechanical tension, thermal denaturation or proteolytic cleavage have been shown to expose functional cryptic elements containing amino acid sequences such as arginine-glycine-aspartic acid (RGD) that allow binding to additional integrins including $\alpha v\beta3$, which is not classically considered a collagen receptor (Davis, 1992; Montgomery, Reisfeld, & Cheresch, 1994). Taken together, these diverse integrins provide the cells with the ability to bind many different types of intact triple-helical collagen, denatured collagen or proteolyzed collagen fragments with both selective and overlapping binding specificities.

The diversity and specificity of downstream signaling stimulated by a particular collagen molecule depend in part on which receptors are engaged as well as the precise composition of the focal adhesion proteins that are assembled following collagen-receptor interaction. Estimates have suggested that over 150 different focal adhesion proteins can participate in the formation of these multi-protein complexes (Zaidel-Bar, Itzkovitz, Ma'ayan, Iyengar, & Geiger, 2007). Depending on the precise combinations of focal adhesion molecules assembled, the cells are provided with the ability to sense the diversity of molecular information found outside the cell and rapidly interpret and integrate the dynamic changes occurring within its local microenvironment to facilitate appropriate responses in a cell type and context-dependent manner.

In addition to integrins, other nonintegrin cell surface molecules have been identified that mediate collagen dependent signaling. Some examples of these collagen receptors include the interesting family of receptor tyrosine kinases called discoidin domain receptors (DDR1 and DDR2), which have been suggested to interact with specific collagen sequences including GVMGFO within the intact triple-helical molecules (Fu et al., 2013; Valiathan, Marco, Leitingner,

Kleer, & Fridman, 2012). Depending on the particular cell type, collagen binding to DDRs may initiate downstream signaling that can have opposing functions such as either stimulating or inhibiting cell migration (Fu et al., 2013; Valiathan et al., 2012). Other examples of cell surface molecules that mediate distinct downstream signaling events following engagement of collagen include glycoprotein-VI (GPVI) and leukocyte-associated immunoglobulin-like receptor (LAIR). GPVI is predominately expressed in platelets and is thought to interact with the immune receptor tyrosine-based activation domains of Fc-receptor- γ and facilitate downstream signaling events associated with Ca^{2+} fluxes and platelet aggregation (Marjoram et al., 2014; Rayes, Watson, & Nieswandt, 2019). In contrast, LAIR-1 (CD305) is broadly expressed in many types of immune cells and specifically contains an immune receptor tyrosine-based inhibitory-like domain. Following collagen binding through GPO-like sequences, LAIR may stimulate immune-suppressive functions in a variety of cells including T cells, dendritic cells, and NK cells (Meyaard, 2008; Rygiel, Stolte, de Ruiter, van de Weijer, & Meyaard, 2011). In fact, studies have suggested that LAIR may function as an immune checkpoint molecule similar to that of CTLA-4 and PD-1/PD-L1 (Meyaard, 2008; Rygiel et al., 2011). These and many other studies implicate collagen in regulating immune cell functions.

Finally, another interesting example of a family of receptors capable of binding collagen include the mannose receptor family. Two distinct members of this family have been documented to bind collagen including the mannose receptor CD206, which is predominately expressed in macrophages and the uPARAP/Endo-180 receptor which has been observed in some epithelial cells, stromal cells including fibroblasts, and selected tumor cells (Melandar, Jurgensen, Madsen, Engelholm, & Behrendt, 2015; Scodeller et al., 2017). A critical function of these cell surface collagen-binding molecules involves internalization of collagen during ECM turnover.

4 | THE GENERATION OF CRYPTIC COLLAGEN ELEMENTS

The triple-helical structure of the intact collagen molecule can be altered in a number of ways that may result in the generation or exposure of normally hidden or cryptic elements (Figure 3) that once exposed, have unique biological functions that may differ from their intact parental molecules. For example, cryptic elements of collagen can be generated by proteolysis of pre-existing fibers that occur outside of the cell (Figure 3a). Alternatively, cryptic collagen elements might also be generated inside the cell as a result of intracellular lysosomal proteases and then subsequently secreted outside the cell in a bioactive form (Figure 3b). Finally, it is also possible that cryptic collagen elements might be generated outside the cell as a result of nonproteolytic mechanisms that are associated with mechanical tension and stretching of collagen molecules (Figure 3c).

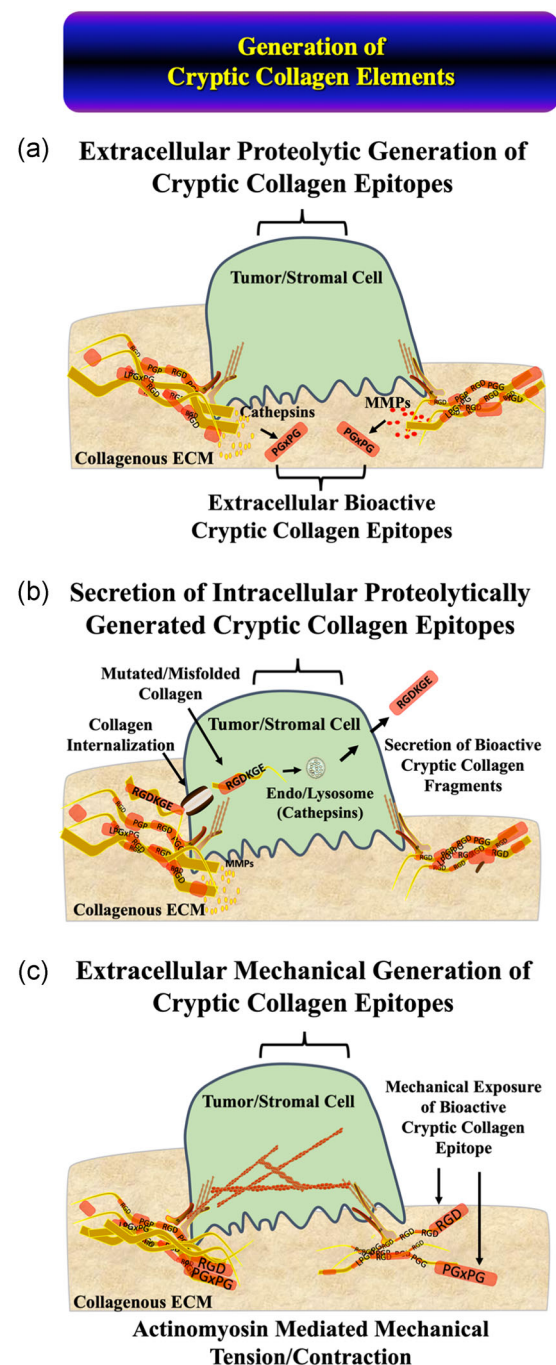


FIGURE 3 Potential mechanisms for the generation of cryptic collagen elements. Schematic representation of three potential mechanisms by which cryptic collagen elements can be generated. (a) Example of the generation of cryptic collagen epitopes mediated by proteolytic remodeling of pre-existing extracellular collagen matrix. (b) Example of the generation of cryptic collagen epitopes from receptor-mediated internalized collagen or mutated or misfolded collagen that is subsequently proteolytically processed then secreted as a small fragment with biological activity. (c) Example of the generation of cryptic collagen epitopes generated by applying cell-mediated mechanical tension or contraction thereby unwinding or altering the triple-helical collagen structure

The terms matrikine and matricryptin have been coined to describe small bioactive peptides or fragments of ECM molecules that exhibit distinct functions as compared to their intact parent protein (Ricard-Blum & Salza, 2014; Ricard-Blum & Vallet, 2016). A rapidly growing subgroup of these matrikine-like molecules (Ames et al., 2016; Colorado et al., 2000; Cretu et al., 2007; Gunda, Boosani, Verma, Guda, & Sudhakar, 2012; Kamphaus et al., 2000; Karagiannis & Popel, 2007; Koskimaki et al., 2010; Lambert et al., 2018; Lindsey et al., 2015; Maeshima et al., 2001; O'Reilly et al., 1997; Ortiz-Urda et al., 2005; Park & Scherer, 2012; Patel & Snelgrove, 2018; Ramchandran et al., 1999; Ricard-Blum & Salza, 2014; Ricard-Blum & Vallet, 2016; Weckmann et al., 2012; J. Xu et al., 2001; R. Xu et al., 2001) include small fragments of genetically distinct types of collagen (Table 1). Many of these collagen-based matrikine-like molecules have been shown to exhibit antitumorigenic activity largely due to their antiangiogenic properties (Colorado et al., 2000; Gunda et al., 2012; Kamphaus et al., 2000;

Karagiannis & Popel, 2007; Koskimaki et al., 2010; Lambert et al., 2018; Maeshima et al., 2001; O'Reilly et al., 1997; Ramchandran et al., 1999; Weckmann et al., 2012; J. Xu et al., 2001). Some examples of the more well-studied antitumorigenic matrikine-like molecules include specific collagen subdomains such as endostatin, arresten, canstatin, tumstatin, restin, hexastatin, pentastatin, and vastatin (Colorado et al., 2000; Gunda et al., 2012; Kamphaus et al., 2000; Koskimaki et al., 2010; Maeshima et al., 2001; O'Reilly et al., 1997; Ramchandran et al., 1999; R. Xu et al., 2001). In contrast, a comparatively smaller number of matrikine-like molecules have been described that exhibit protumorigenic activity including the cryptic collagen elements termed HUIV26, HU177, XL313, and endotrophin (Ames et al., 2016; Cretu et al., 2007; Park & Scherer, 2012; J. Xu et al., 2001). These unique cryptic collagen elements can be generated as either soluble matrikine-like fragments or as structurally altered regions of collagen that remain as a solid phase element immobilized within the ECM (Ames et al., 2016;

Cryptic collagen element	Molecular origin	Impact tumor/angiogenesis	References
Endostatin	Coll type-XVIII	Inhibitory	O'Reilly et al. (1997)
Arresten	Coll type-IV	Inhibitory	Colorado et al. (2000)
Restin	Coll type-XV	Inhibitory	Ramchandran et al. (1999)
Canstatin	Coll type-IV	Inhibitory	Kamphaus et al. (2000)
Tumstatin	Coll type-IV	Inhibitory	Maeshima et al. (2001)
Hexastatin	Coll type-IV	Inhibitory	Gunda et al. (2012)
Pentastatin	Coll type-IV	Inhibitory	Koskimaki et al. (2010)
Vastatin	Coll type-VIII	Inhibitory	R. Xu et al. (2001)
Tetrastatin	Coll type-IV	Inhibitory	Lambert et al. (2018)
Lamstatin	Coll type-IV	Inhibitory	Weckmann et al. (2012)
a6(IV) NC1	Coll type-IV	Inhibitory	Karagiannis and Popel (2007)
a4(IV) peptide	Coll type-IV	Inhibitory	Karagiannis and Popel (2007)
a5(IV) peptide	Coll type-IV	Inhibitory	Karagiannis and Popel (2007)
FNCI	Coll type-VII	Inhibitory	Ortiz-Urda et al. (2005)
N-ac PGP peptide	Coll type-I	Stimulatory?	Patel and Snelgrove (2018)
PGP peptide	Coll type-I	Stimulatory?	Patel and Snelgrove (2018)
HUIV26	Coll type-IV	Stimulatory	J. Xu et al. (2001)
HU177	Multiple coll types	Stimulatory	Cretu et al. (2007)
XL313	Coll type-I	Stimulatory	Ames et al. (2016)
Endotrophin	Coll type-VI	Stimulatory	Park and Scherer (2012)
C-1158/59	Coll type-I	Stimulatory	Lindsey et al. (2015)

TABLE 1 Representative examples of selected cryptic collagen elements that have been shown to differentially impact angiogenesis and tumor growth

Park & Scherer, 2012). Additional matrikine-like fragments derived from collagen that contains the PGP amino acids have also been shown to be potent chemotactic factors for immune cells such as neutrophils (Patel & Snelgrove, 2018). Given that PGP peptides are thought to play roles in recruiting neutrophils to areas of active infections and inflammation, it is likely that a tightly controlled balance of the relative levels of these collagen PGP peptides is critical to maintaining functional homeostasis, as either prolonged neutrophil stimulation or conversely, insufficient neutrophil recruitment may cause tissue damage or impair an effective response to infection. Interestingly, recent studies have shown that collagen-derived acetylated proline-glycine-proline peptide (N- α -PGP) can be detected at elevated levels in the plasma of subjects diagnosed with chronic pulmonary lung disease (Hahn et al., 2015; Weathington et al., 2006). These collagen-derived peptides were also shown to enhance endothelial permeability and cause vascular leakage that was dependent on signaling through CXCR2, which in turn led to enhanced phosphorylation of Erk and PAK, which are known to regulate angiogenesis (Hahn et al., 2015; Weathington et al., 2006). Given these data and the role of neutrophils in regulating angiogenesis and tumor growth, it would not be surprising that a subpopulation of these PGP-containing matrikines might also play active roles in tumor growth and metastasis.

Among the most well-studied and physiologically relevant mechanisms that contribute to the generation of cryptic collagen elements include intracellular and extracellular proteolysis as well as mechanical forces such as cell-mediated tension and contraction (Figure 3a–c). It is well established that the triple-helical structure of collagen results in it being highly resistant to most proteolytic enzymes. Two important families of proteases which have the capacity to cleave intact triple-helical collagen include members of the matrix metalloproteinases (MMPs) and cathepsin families (Kessenbrock, Plaks, & Werb, 2010; Olson & Joyce, 2015). Matrix metalloproteinases comprise a class of at least 28 different zinc-dependent enzymes with distinct and sometimes overlapping substrate specificities. Members of the MMP family can be either secreted or membrane-associated and have been suggested to have both catalytic as well as noncatalytic functions (Kessenbrock et al., 2010). Members of the MMP family are generally composed of three-distinct domains including a pro-domain that helps keep the enzymes in a catalytically inactive state, a catalytic domain that is rendered functional following structural alterations of the pro-domain, and a hemopexin-like domain that contributes to substrate binding specificity. Examples of MMPs shown to cleave intact triple-helical collagen into $\frac{1}{4}$ and $\frac{3}{4}$ size fragments include MMP-1, -2, -3, -8, -13–16 (Kessenbrock et al., 2010). Once the triple-helical collagen structure has been proteolyzed or structurally denatured, additional enzymes such as the gelatinases including MMP-2 and MMP-9 can further degrade the denatured collagen into smaller fragments. In this regard, studies have shown these MMPs are functionally involved in regulating the generation of collagen-based-matrikine-like molecules in both a positive and negative fashion (Wells, Gaggari, & Blalock, 2015). MMPs have been shown to help generate the antiangiogenic molecule tumstatin while blocking MMPs were shown to limit its generation (Hamano et al., 2003). In contrast, MMPs such as MMP-9 have also been shown to help expose the HU177 cryptic elements within collagen

(Hangai et al., 2002). These studies help illustrate how MMPs may exhibit both protumorigenic as well as antitumorigenic activities depending on the specific circumstances.

The biological functions of MMPs as they relate to tumor growth and metastasis are complicated as examples of opposing roles of MMPs in tumor growth have been documented (Kessenbrock et al., 2010; Wells et al., 2015). Reports on the differential roles of MMPs on tumor growth and metastasis might be explained at least in part, by the fact that certain MMPs might promote the generation of antiangiogenic matrikines, while in other cases, MMPs may actively generate protumorigenic cryptic collagen elements that enhance tumor progression. Collagen remodeling is also known to occur during normal physiological processes such as ovulation, normal postpartum mammary gland involution, and wound healing (Curry & Smith, 2006; O'Brien et al., 2010; Sheets et al., 2016). Thus, the precise timing of when distinct proteinases are expressed and activated, along with their tissue localization and differential substrate specificity, likely contributes to their differential impact during normal physiological processes as well as tumor progression. To this end, a therapeutic strategy that broadly targets all MMPs may have limited therapeutic utility and will likely require specialized approaches to use this strategy effectively in the clinical setting.

As mentioned above, additional enzymes such as cathepsins have also been shown to cleave and generate cryptic ECM fragments including those of collagen. Although the majority of MMP-mediated proteolytic activity is thought to occur largely outside of the cell within the extracellular or peri-cellular space, cathepsin-dependent proteolytic activity can occur both outside as well as inside the cell. For example, cathepsins such as cathepsin K, L, and S have been shown to exhibit proteolytic functions outside the cell and help generate bioactive ECM fragments (Olson & Joyce, 2015; Veillard et al., 2011). The generation of these matrikine-like molecules may in turn bind to specific cell surface receptors, thereby helping to regulate tumor growth and metastasis. However, it is also possible that intracellular lysosomal cathepsins can generate cryptic fragments of collagen inside the cell (Ishida & Nagata, 2009; Wagenaar-Miller et al., 2007). Interestingly, using novel zebrafish models and electron microscopy imaging, investigators have provided new evidence that invasion through basement membranes by macrophages may involve degradation of collagen and subsequently internalization of this collagen as these macrophages are recruited towards pre-malignant cells (Van den Berg et al., 2019). These data along with other intracellular mechanisms of collagen degradation are gaining significant attention as remodeled fragments of collagen have been shown to play critical roles in a number of pathological processes including angiogenesis, tumor growth, metastasis, and inflammation. Interestingly, CD206 is upregulated in alternatively activated M2-like macrophages, which are thought to play important roles in normal physiological events. In addition, M2-like macrophages also are thought to play a role in pathological processes such as malignant tumor growth (Scodeller et al., 2017). These studies provide evidence of a potential mechanism by which small fragments of collagen can be generated within the

local tissue microenvironment under both normal and pathological conditions. A second mannose receptor family member uPARAP/Endo-180 has also been shown to specifically internalize collagen for further cathepsin-mediated proteolytic degradation in lysosomes of stromal cells such as fibroblasts (Melander et al., 2015). The molecular mechanisms associated with collagen degradation as well as both conventional and unconventional protein secretion are becoming more well-defined within the endo-lysosomal compartments following ER stress, and include the unfolded protein response, ER-associated degradation (ERAD) and autophagic/lysosomal associated mechanisms, which collectively contribute to degrading and eliminating misfolded molecules (Gotkin, Ripley, Lamande, Bateman, & Bienkowski, 2004; Ishida & Nagata, 2009; Lee & Ye, 2018; Ponpuak et al., 2015). Given these studies, and the growing appreciation of the importance of unconventional protein secretion pathways such as secretory autophagy (Ponpuak et al., 2015) and misfolding-associated protein secretion (MAPS) in regulating many biological events, these processes may represent previously unrecognized mechanisms by which small collagen fragments can be actively secreted out of cells in a bioactive form (Ponpuak et al., 2015).

Interestingly, studies have shown that newly synthesized collagen that is either mutated or misfolded can lead to rapid accumulation of abnormal aggregates that result in ER stress, which subsequently facilitate either autophagy-lysosomal mediated degradation or ERAD (Anelli & Sitia, 2008; Gotkin et al., 2004; Ishida & Nagata, 2009; Lee & Ye, 2018; Ponpuak et al., 2015). These active intracellular processes might, in turn, generate bioactive cryptic collagen fragments. It would be interesting to speculate that certain forms of these intracellular-derived cryptic collagen fragments may be actively secreted outside cells in a form that regulates tumor growth and metastasis. In fact, studies have previously described functional mechanisms of unconventional protein secretion of different leaderless proteins and proteolytically altered molecules such as IL-1 α , IL-1 β , IL-33 HMGB1, FAB4, and vWF which exhibit biological activity (Anelli & Sitia, 2008; Torisu et al., 2013; Villeneuve et al., 2018). Thus, a rapidly accumulating body of evidence now indicates that unique secretory mechanisms such as secretory autophagy and MAPS may provide biologically relevant mechanisms to bypass the conventional ER-Golgi secretory pathways leading to the release of biologically active molecules.

Finally, in addition to proteolytic mechanisms that contribute to the generation of cryptic collagen elements, mechanical forces such as stress, tension, and/or contraction applied to proteins may also play biologically important roles in the generation of cryptic ECM epitopes. Recent biophysical studies have suggested that enhanced mechanical tension on collagen can lead to alterations at the molecular level leading to changes in triple-helical structure (Adhikari, Glassey, & Dunn, 2012; Flynn et al., 2010). In fact, some studies indicate that high mechanical tension can change the molecular architecture by uncoiling the molecule and placing it in a stably denatured form with enhanced susceptibility to proteolytic cleavage (Adhikari et al., 2012). In contrast, other studies have suggested that enhanced mechanical strain through stretching individual collagen

fibers may alter its structure in such a way as to cause reduced proteolytic degradation (Flynn et al., 2010). Thus, changes in mechanical tension can directly affect the rate of proteolytic degradation either positively or negatively by altering the mechanochemistry of the collagen triple-helical molecule. These mechanical changes would likely expose unique cryptic regions of the molecule that may stimulate different signaling pathways. Whether changes in mechanical forces increase or decrease proteolytic degradation likely depends on a host of different parameters including the type of proteases, and the presence or absence of specific proteins bound to the collagen within the specific tissue microenvironments. In any event, mechanical forces acting on the collagen triple-helical molecule may efficiently alter its overall structure, which in turn, could result in exposure of cryptic epitopes or regions that were normally hidden.

Recent studies have shown that local micro-unfolding of the collagen molecule due to mechanical tension may occur (Glentis et al., 2017; Spaderna et al., 2006). Studies have also shown the presence of pre-existing small pores and structural defects in the basement membranes of colorectal tumors and the presence of these structurally altered basement membranes correlated with enhanced metastasis and poor survival of subjects with colorectal cancer (Spaderna et al., 2006). Given that oscillatory mechanical forces generated at cell-ECM contact points can result in changes in the tensile forces, it would be interesting to speculate that these types of transient changes in mechanical forces might be sufficient to cause micro-perforations and or unfolding of collagen triple-helical regions of the basement membranes, which have at times been observed in vivo (Glentis et al., 2017). For example, small micro-perforations formed within the basement membrane of epithelial sheets during branching morphogenesis in the salivary gland as well as bronchial epithelium have been described and may be associated with both protease dependent or mechanical actinomycin-dependent changes (Harunaga, Doyle, & Yamada, 2014). These structural changes, in turn, may alter the biomechanical parameters of the basement membrane making it more deformable. Another example is illustrated in lymphatic vessels of the skin, as pre-existing micro-holes and/or perforations within collagen-IV containing basement membranes exist through which leukocytes such as dendritic cells migrate (Pflücke & Sixt, 2009). These basement membranes were characterized by discontinuous layers of collagen type-IV (Pflücke & Sixt, 2009). The size of the holes or perforations changed with time, suggesting the possibility that physical changes in the geometry and structure of collagen can occur rapidly by protease dependent or independent mechanisms. In support of nonproteolytic mechanisms for structurally altering basement membranes, intriguing new studies showed that biopsies from human colon cancers exhibited areas enriched with α SMA-positive cancer-associated fibroblast (CAF)-like cells that were colocalized with regions of the basement membrane that were structurally defective or discontinuous (Glentis et al., 2017; Spaderna et al., 2006). Interestingly, CAF-like cells isolated from human subjects with colon cancer were shown to actively exert mechanical tension and stretching on the basement membrane via physical

actinomyosin-associated contraction forces, resulting in the CAFs pulling and stretching the basement membrane molecules (Glentis et al., 2017). These cellular processes were thought to reorganize the overall structure of collagen allowing cancer cells to breach through the basement membrane thereby promoting invasion (Glentis et al., 2017). Finally as mentioned previously, studies have suggested that WISP1/CNN4 can be expressed by certain tumor cells, which can in turn bind to collagen, and slow down fibrillogenesis, thereby causing collagen fiber linearization (Jia et al., 2019). Although it is unclear whether this process results in generation of discrete functional cryptic collagen epitopes, the linearization of collagen in this setting promotes tumor cell metastasis (Jia et al., 2019). Thus, it would be interesting to speculate that certain nonproteolytic biophysical and biomechanical changes in collagen structure might expose unique cryptic sites within collagen molecules either permanently or transiently, thereby allowing temporal initiation of signaling pathways at specific tissue locations to potentiate cancer cell invasion.

5 | FUNCTIONAL IMPLICATIONS OF ALTERED COLLAGEN STRUCTURE ON TUMOR GROWTH AND METASTASIS

The diversity of functions mediated by collagen can depend on the cells' ability to bind to discrete regions of collagen made up of different amino acid sequences. The specificity of integrin binding to ECM proteins and subsequent downstream signaling events can be altered, depending on the precise molecular orientation of the contact residues and specific amino acids engaged by cell surface receptors. Thus, the precise sequence of amino acids as well as how they are geometrically oriented in space can help fine-tune which specific cell surface receptors are activated. In turn, the specific repertoire of activated receptors can further differentiate the signaling programs stimulated downstream of ECM engagement, thereby providing the cells with a wide array of different molecular outputs as the cells actively sample different local tissue microenvironments. For example, elegant studies have demonstrated that bone marrow-derived mesenchymal cell interactions with denatured collagen as opposed to intact collagen stimulated a signaling program that facilitates differentiation into an adipocyte-like phenotype, while in contrast, cellular interaction with intact collagen promoted signaling leading to more efficient osteogenic differentiation (Mauney & Volloch, 2009). These important studies indicate that cellular interactions with cryptic regions of collagen can function as unique signaling hubs that initiate distinct pathways that promote complex differentiation programs.

As suggested above, collagen can have both stimulatory and inhibitory effects on tumor progression, which can be governed by its overall structural geometry. Collagen found surrounding normal breast epithelial tissues tends to have a random array of fibers with a curly appearance (Conklin et al., 2011). In contrast, thicker fibrils with a more parallel linear arrangement with enhanced mechanical

stiffness have been suggested to promote tumor growth and metastasis (Conklin et al., 2011). In fact, investigators have developed a set of morphological and structural parameters of collagen organization that may help in distinguishing the malignant features of some types of tumors (Conklin et al., 2011). Studies have also suggested that the dense networks of collagen molecules may represent a physical barrier for migration of both tumor cells and immune cells and thus the overall collagen architecture may govern both the extent and direction of movement of tumor cells as well as control the infiltration of stromal cells such as cancer-associated fibroblasts and both adaptive and innate immune cells into tumors. In fact, recent studies have suggested that activated T cells may get stuck within the dense and highly collagenous stroma surrounding some tumor types, thus limiting their infiltration and subsequent immune-mediated control of tumor growth (Kaur et al., 2019; Mariathasan et al., 2018).

Mounting evidence for the critical roles of collagen remodeling in the differential control of both stromal and tumor cell behavior is emerging. A well-established hallmark of tumor progression involves ECM remodeling and a wealth of data indicates multiple roles of collagen in facilitating tumor progression. However, given that collagen is the most abundant ECM protein in the body, directly blocking all cellular interactions with intact triple-helical collagen as a therapeutic strategy would likely have significant adverse events. In this regard, investigators have examined multiple approaches to selectively disrupt collagen-mediated signaling such as by targeting collagen specific cell surface receptors including integrins and DDRs.

An alternative strategy that would likely provide a significant degree of additional specificity would be to selectively target functional epitopes of structurally altered forms of ECM proteins that serve as ligands for these ECM receptors, instead of direct targeting of the ECM receptors themselves. These cryptic elements in conjunction with their associated receptors can be considered biologically active cryptic signaling hubs. Importantly, if site-specific generation of cryptic elements occurs during pathological processes, and in turn, if cellular interactions with these cryptic elements play functional roles in controlling crucial signaling cascades, then directly targeting these cryptic signaling hubs might provide a unique strategy to selectively disrupt site-specific signaling events that regulate angiogenesis, tumor growth and metastasis (Figure 4). However, it is also important to point out that certain cryptic collagen elements may also be generated during normal physiological events including wound healing, thus a clear understanding of possible roles of cryptic collagen elements in normal physiological processes is important when designing therapeutic strategies. In this regard, some of the earliest evidence indicating the presence of cryptic collagen elements hidden within the three-dimensional structure of collagen was provided by the pioneering work of Davis and others, which showed that the RGD binding integrin $\alpha\beta3$ could selectively bind to proteolyzed and denatured collagen while exhibiting little if any binding to intact collagen (Davis, 1992). Elegant studies by Montgomery and colleagues provided some of the first functional evidence showing that disrupting $\alpha\beta3$ -mediated cellular interactions with these cryptic collagen elements could selectively regulate apoptosis in melanoma

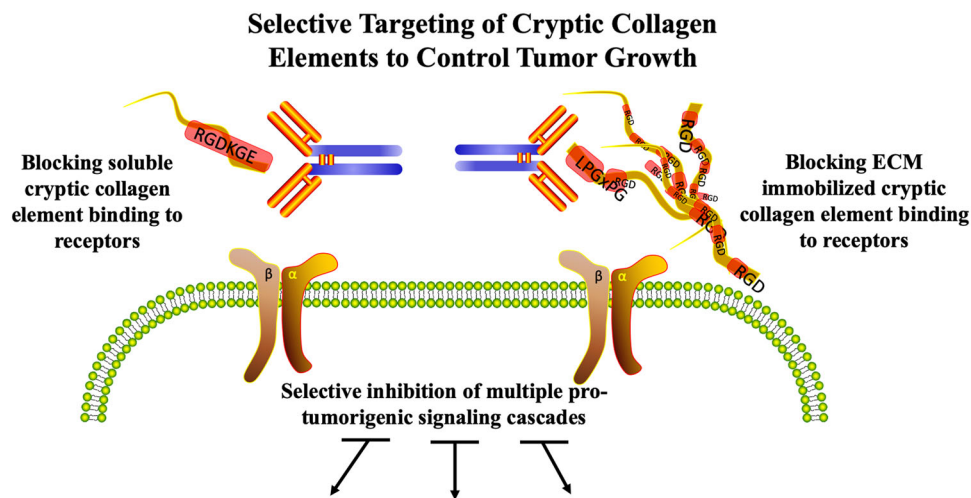


FIGURE 4 Targeting cryptic collagen elements. During tumor growth and metastasis, cryptic collagen epitopes can be generated by multiple mechanisms. Once generated, these cryptic collagen epitopes may initiate protumorigenic and/or immunosuppressive signaling pathways within malignant cells as well as within various stromal cell types that collectively promote tumor growth and metastasis. Blocking cellular interactions with the cryptic collagen epitopes by targeting the cryptic collagen epitopes themselves may represent a highly effective way to selectively inhibit protumorigenic signaling cascades in multiple cell types that contribute to tumor growth and metastasis

cells (Montgomery et al., 1994). Taken together, these studies opened up new avenues of research into whether selective targeting of cryptic collagen elements might provide effective new strategies to control tumor growth.

In order for the strategy of selective targeting of cryptic collagen elements to be translated into clinically useful treatment paradigms, strong evidence is needed to establish that cryptic collagen elements are indeed generated *in vivo* during tumor development. Studies using both murine and human tumors and the corresponding normal tissues have indicated the preferential exposure of cryptic collagen elements within the ECM of multiple tumor types *in vivo*. For example, Mab HUIV26 was shown to selectively bind to a cryptic collagen element generated specifically within collagen type-IV following proteolysis or thermal denaturation but failed to bind to intact collagen type-IV or intact or denatured forms of other types of collagen (J. Xu et al., 2001). The cryptic collagen element recognized by this antibody was generated within the basement membranes of tumor-associated blood vessels, but not normal quiescent vessels, confirming its selective generation *in vivo* (J. Xu et al., 2001). In further studies, a different antibody termed Mab HU177 was shown to selectively bind to a set of different cryptic collagen epitopes present within many different types of collagen, including collagen type-I, -II, -III, -IV and -V, but failed to bind normal intact triple-helical collagen (Cretu et al., 2007; Freimark et al., 2007). This antibody was shown to bind to a repetitive and highly conserved collagen sequence containing the amino acids PGxPG, where x can represent multiple amino acids within collagen (Cretu et al., 2007; Freimark et al., 2007). The cryptic collagen epitope recognized by the Mab HU177 was selectively exposed within the ECM of many histologically distinct tumor types and was observed within both the basement membrane compartments as well as the interstitial ECM, but exhibited minimal

if any expression within normal quiescent tissues (Cretu et al., 2007; Pernasetti et al., 2006). Further evidence of the selective generation of cryptic collagen elements comes from elegant studies from Liu and others (Hwang et al., 2017) who developed an interesting set of peptide reagents designed to selectively bind denatured forms of collagen (Hwang et al., 2017). Using these unique denatured collagen-binding peptides, investigators have shown generation of denatured forms of collagen within tissues undergoing ECM remodeling (Hwang et al., 2017). Moreover, additional studies have provided evidence for the generation of a C-terminal cleavage fragment of the $\alpha 3$ chain of collagen type VI, which has been termed endotrophin (Park & Scherer, 2012). Interestingly, endotrophin was shown to be generated by stromal adipocytes-like cells within the stroma of tumors and may regulate angiogenesis and tumor progression (Park & Scherer, 2012). Taken together, these and other studies provide compelling evidence for the selective generation of cryptic collagen elements within the matrix of tumors, and importantly, these cryptic collagen epitopes might be targeted by both peptide and antibody reagent *in vivo*.

6 | THERAPEUTIC TARGETING OF CRYPTIC COLLAGEN ELEMENTS

To examine whether the generation of structurally altered collagen sites plays a functional role in controlling cellular behavior, studies were carried out to examine the impact of directly targeting the HUIV26 cryptic collagen elements on tumor growth (J. Xu et al., 2001). Early studies using anti-HUIV26 Mab provided the first direct evidence that cells could selectively bind to the HUIV26 cryptic collagen element exposed within denatured collagen type-IV,

as Mab HUIV26 blocked cell adhesion and migration on denatured collagen, but had no effect on adhesion or migration of cells on intact triple-helical collagen (J. Xu et al., 2001). These studies indicate that cellular interactions with the HUIV26 collagen epitope can actively control the adhesive and migratory behavior of cells which bind to structurally altered collagen type-IV. Surprisingly, cellular interaction with the HUIV26 epitope was shown to be mediated by $\alpha\text{v}\beta 3$ in an RGD-independent manner (J. Xu et al., 2001).

Given that cellular interaction with the HUIV26 epitope was shown to be mediated by integrin $\alpha\text{v}\beta 3$, and that $\alpha\text{v}\beta 3$ regulates Fak/Src-dependent signaling cascades that modulate endothelial and tumor cell behavior, studies were next undertaken to examine the effects of selectively targeting the HUIV26 collagen epitope on angiogenesis. Using a combination of mouse, rat, and chick embryo animal models, systemic administration of an anti-HUIV26 cryptic collagen epitope Mab potently inhibited growth factor-induced angiogenesis in vivo (J. Xu et al., 2001). Further studies using an ischemia-induced retinal neovascularization model (J. Xu et al., 2001) or a model of thymic neovascularization (Odaka, Tanioka, & Itoh, 2005), indicated that direct targeting of the HUIV26 collagen epitope also exhibited potent antiangiogenic activity in vivo. Finally, a humanized version of Mab HUIV26 termed H8, also demonstrated inhibitory activity in a mouse model of choroidal neovascularization that occurs during macular degeneration (Jo et al., 2006). Given the role of angiogenesis in facilitating tumor growth, further studies were performed to examine the role of the HUIV26 collagen epitope on tumor growth. Systemic administration of Mab HUIV26 inhibited HT1080 fibrosarcoma and CS1 melanoma tumor growth in the chick embryo model by approximately 50%, while human M21 melanoma tumor growth in SCID mice was inhibited by over 75% (J. Xu et al., 2001). When taken together, these early studies provide the first evidence that a therapeutic strategy of directly targeting a cryptic collagen element selectively exposed within the ECM of tumors may represent an effective approach to control malignant tumor growth.

A second example of a functional cryptic collagen epitope that represents a therapeutic target can be illustrated by the highly conserved PGxPG-containing HU177 cryptic collagen element that is exposed within multiple types of collagen following structural remodeling. Interestingly, in contrast to the HUIV26 epitope, which is recognized by integrin $\alpha\text{v}\beta 3$, the collagen-binding integrin $\alpha 10\beta 1$ was shown to serve as the major receptor for the HU177 epitope, however, some binding was also detected by integrin $\alpha 2\beta 1$ (Caron et al., 2016). An antibody directed to the HU177 collagen epitope potently inhibited the ability of endothelial cells, CAF-like cells as well as tumor cells to bind to denatured collagen and migrate, but failed to inhibit cellular binding or migration on intact collagen (Caron et al., 2016). These studies provide evidence for a second functional cryptic collagen element hidden within multiple types of collagen. Initial studies using anti-HU177 Mab demonstrated its ability to potently inhibit angiogenesis induced by purified growth factors such as FGF2 in the chick CAM model as well as inhibit tumor-associated angiogenesis in both the chick embryo and mouse

models using a number of distinct tumor types including, melanomas, ovarian carcinomas and breast carcinomas (Caron et al., 2016; Caron, Han, Contois, Vary, & Brooks, 2018; Pernasetti et al., 2006). Blocking endothelial cell interaction with the HU177 collagen element resulted in enhanced levels of the cyclin-dependent kinase inhibitor P27KIP1, consistent with a role for the HU177 collagen element in regulating P27KIP1 activity (Cretu et al., 2007). In further studies, direct targeting of the HU177 cryptic collagen element with the humanized Mab D93 inhibited mammary tumor growth in mice and enhanced the antitumor activity of Taxol (Pernasetti et al., 2006).

Given the preclinical antiangiogenic and antitumor activity of the humanized version of Mab HU177, a phase-I human clinical trial was carried out. Results from this first human study were encouraging as no dose-limiting toxicities were observed and 26% of the treated patients showed evidence of clinical responses including stable disease (Robert et al., 2010). Interestingly, a subject with ovarian cancer exhibited a mixed response with shrinkage of a metastatic liver lesion (Robert et al., 2010). Although this human clinical study provided the first evidence that selectively targeting a cryptic collagen epitope may represent a new clinical strategy for the treatment of malignant tumors, relatively little was known concerning the potential cellular and molecular mechanisms that contribute to the antitumor activity. To this end, more detailed cellular and molecular studies were carried out to gain a more in-depth mechanistic understanding of the functional roles of the HU177 cryptic collagen epitope in tumor growth and metastasis. Interestingly, in addition to inhibiting endothelial cell migration and angiogenesis, new studies have now indicated that targeting of the HU177 cryptic collagen element selectively inhibited CAF-like cell migration on denatured collagen in vitro and this migratory activity appeared to depend in part on growth factor-stimulated activation of MAPK/Erk signaling, as Mab D93 selectively inhibited growth factor-induced migration of CAF-like cells on denatured collagen as well as inhibited downstream Erk phosphorylation (Caron et al., 2016). Surprisingly, Mab D93 also inhibited infiltration of CAF-like cells into SKOV-3 ovarian tumors growing in mice by nearly 70% (Caron et al., 2016). These data in conjunction with previous studies indicate that in addition to inhibiting angiogenesis, direct targeting the HU177 cryptic collagen element may also selectively limit migration and infiltration of CAF-like cells, which are thought to help provide tumors with many protumorigenic growth factors and cytokines as well as facilitating immune suppression.

Although multiple studies provide evidence for a role for the HU177 epitope in regulating stromal cell migratory behavior, the majority of patients die from cancer as a result of metastatic dissemination of the malignant tumor cells. Recent studies indicate for the first time that direct tumor cell binding to the HU177 cryptic collagen epitope within collagen type-IV results in the initiation of a signaling cascade that reprograms melanoma cells with enhanced capacity to migrate in vitro and metastasize in vivo (Caron et al., 2018). Surprisingly, B16F10 melanoma cell binding to the HU177 cryptic collagen epitope led to enhanced activation and nuclear accumulation of yes-associated protein (YAP) (Caron et al., 2018). The selective

nuclear accumulation of YAP stimulated by binding to the HU177 collagen epitope was dependent on the cyclin-dependent kinase-5 (CDK5), as knock-down of Cdk5 reduced the ability of the HU177 collagen element to stimulate YAP nuclear accumulation (Caron et al., 2018). Moreover, the ability of anti-HU177 Mab to inhibit experimental metastasis depended on inhibiting YAP as Mab D93 failed to inhibit metastasis of melanoma cells lacking YAP (Caron et al., 2018). Collectively, these studies not only help define novel downstream integrin-dependent signaling cascades following binding to the HU177 cryptic collagen element but may help in optimizing the clinical strategy of targeting the HU177 collagen epitope.

Further studies have provided additional evidence that selective targeting of cryptic collagen elements may represent an effective new therapeutic strategy to treat angiogenic diseases. For example, Mueller and colleagues used a unique phage display approach to isolate a tumor homing peptide that selectively bound to MMP-2 cleaved collagen type-IV (Mueller, Gaertner, Blechert, Janssen, & Essler, 2009). Interestingly, this unique peptide selectively bound to MMP-2 cleaved collagen type-IV but not to intact collagen or to collagen cleaved by other proteases such as MMP-9 (Mueller et al., 2009). This unique binding specificity suggested that this particular cryptic collagen epitope was different than either the HUIV26 or HU177 epitopes described previously. Importantly, this peptide was shown to inhibit endothelial cord formation *in vitro* and angiogenesis *in vivo* (Mueller et al., 2009). Finally, an additional example of the potential importance of directly targeting cryptic collagen fragments to control pathological processes includes direct targeting of endotrophin, the C-terminal cleavage fragment of the $\alpha 3$ chain of collagen VI. In this regard, studies have shown that this collagen fragment can help regulate, angiogenesis, tumor growth, and metastasis, and importantly, direct targeting of this collagen fragment was shown to inhibit angiogenesis and tumor growth *in vivo* (Park & Scherer, 2012). Taken together, these studies suggest that multiple cryptic collagen epitopes may exist within the ECM and may represent important new therapeutic targets for the treatment of tumors.

Although a subset of cryptic collagen elements has been shown to be selectively generated during tumor growth, it is also important to evaluate the overall strategy of therapeutic targeting of cryptic collagen elements with caution, given the possibility that certain cryptic elements may play functional roles in normal tissue remodeling. Moreover, collagen remodeling is thought to play an important role in ovulation, as investigations have shown significant alterations in the integrity of intact collagen during follicular rupture that is required for the appropriate ovulation to occur (Curry & Smith, 2006). Thus, it is possible that direct targeting of cryptic collagen epitopes may adversely impact ovulation depending on the complement of cell surface receptors available to mediate the signaling from the cryptic collagen fragments.

In further studies, normal postpartum mammary gland involution was shown to be associated with significant collagen remodeling (O'Brien et al., 2010). Interestingly, M2-like macrophages were shown to infiltrate the involuting mammary gland, and proteolyzed

collagen along with these M2-like macrophages was thought to play a functional role in converting the involuting mammary gland phenotype back to a normal quiescent state. Although the exact sequence, structural and functional characteristics of the proteolyzed collagen observed in these studies is not completely understood, these findings suggest that it is possible that direct targeting of cryptic collagen epitopes may interfere with normal postpartum mammary gland involution. Finally, normal wound healing is thought to require the coordinated activity of a number of distinct cell types that may be controlled by interactions with cryptic collagen elements including endothelial cells, fibroblasts, and macrophages. In this regard, specific cryptic collagen epitopes have been shown to play roles in regulating angiogenesis and the behavior of fibroblasts and inflammatory infiltrates. For example, direct targeting of the XL313, HUIV26, and HU177 cryptic collagen elements have been shown to inhibit angiogenesis (Ames et al., 2016; Cretu et al., 2007; J. Xu et al., 2001) and angiogenesis is thought to play a role in wound healing. Moreover, direct targeting of the HU177 collagen epitope was shown to inhibit infiltration of cancer-associated fibroblasts. As mentioned above, the ability of many cryptic collagen epitopes to stimulate cell signaling and alter cellular behavior depends on the expression of cell surface receptors capable of binding to these collagen elements. Thus, the possibility that direct targeting of these functional collagen elements with blocking antibodies or other antagonists that might alter normal physiological processes will likely depend on both generation of the particular epitopes and the contribution of distinct cell types that have the capacity to bind and respond to these cryptic elements.

7 | SOLUBLE CRYPTIC COLLAGEN ELEMENTS AS POTENTIAL THERAPEUTIC TARGETS

Although functional cryptic epitopes selectively exposed within structurally altered regions of collagen that remain immobilized locally within the ECM have been shown to represent therapeutic targets, soluble circulating collagen fragments with unique biological activities may also play a role in governing multiple diseases. To this end, studies have shown strong correlations between the relative levels of circulating proteolytic collagen fragments and tumor progression (Ng et al., 2008). However, the ability of these collagen fragments to specifically impact disease progression either positively or negatively is not completely understood. Additional studies will be required to help define specific receptors for these collagen fragments and uncover possible molecular signaling pathways that they might regulate to better determine the therapeutic utility of targeting these collagen fragments. An important rationale for defining the biological roles of soluble collagen fragments in promoting tumor progression comes from interesting new findings that indicate elevated levels of a subset of specific proteolytic collagen fragments detected in the circulation of cancer patients correlated with resistance to anti-CTLA-4 immune checkpoint therapy (Jensen et al., 2018).

Evidence is beginning to emerge that certain soluble collagen fragments may not only represent biomarkers of enhanced ECM remodeling but actually exhibit direct biological activity that can be selectively targeted to control pathological processes. For example, recent studies have identified a soluble proteolytic fragment of the $\alpha 1$ chain of collagen type-1 (C-1158/59) generated *in vivo* by MMP-2 and MMP-9 was detected in human plasma, and interestingly, the levels of this collagen fragment were shown to be reduced within subjects following myocardial infarction (MI) (Lindsey et al., 2015). Moreover, this collagen fragment was also shown to regulate fibroblast cell migration *in vitro* and angiogenesis, as treatment of mice with a synthetic peptide corresponding to a region of the C-1158/59 collagen fragment increased wound healing, reduced pathological left ventricle dilation, and stiffness as well as fibrosis following MI, which is consistent with the functional activity of this collagen fragment *in vivo* (Lindsey et al., 2015). Given that certain soluble cryptic collagen peptides may play roles in normal physiological processes, it will be critical to use caution when modulating their biological activity by directly targeting them. In this regard, in addition to the important work involving MI, other studies have indicated correlations between the relative levels of the collagen-derived fragment N- α -PGP and inflammatory diseases (Patel & Snelgrove, 2018) and this collagen fragment was shown to induce endothelial permeability and vascular leakage during acute respiratory distress syndrome (Hahn et al., 2015). Importantly, blocking N- α -PGP binding to CXCR2 using a specific peptide antagonist inhibits vascular leakage providing evidence that selectively targeting the N- α -PGP collagen peptide may provide therapeutic benefit (Patel & Snelgrove, 2018). In addition, a soluble RGDKGE containing fragment of collagen termed XL313 that is actively generated and secreted in a soluble form by M2-like macrophages has been described (Ames et al., 2016). The XL313 cryptic collagen fragment was shown to bind to integrin $\alpha\beta 3$ on the surface of endothelial cells and subsequently activate Src and P38MAPKinase (Ames et al., 2016). Stimulation of endothelial cells with a synthetic version of the XL313 collagen fragment resulted in enhanced nuclear accumulation of YAP, increased proliferation *in vitro*, and induction of inflammation and angiogenesis *in vivo* (Ames et al., 2016). Importantly, an antibody specifically directed to the RGDKGE collagen fragment potentially inhibited angiogenesis and inflammation *in vivo* (Ames et al., 2016). These preclinical studies provide additional evidence for the possibility that a strategy that selectively targets soluble cryptic collagen fragments may provide a unique approach to control pathological processes *in vivo*.

Finally, studies by Ng and others provided evidence that the HU177 cryptic collagen epitope can also be released into the circulation in a soluble form (Ng et al., 2008). Elevated levels of a soluble form of the HU177 collagen epitope significantly correlated with primary tumor thickness in a cohort of human subjects diagnosed with melanoma (Ng et al., 2008). As mentioned above, the humanized Mab directed to this HU177 cryptic collagen element (Mab D93) was examined in a phase-I human clinical trial with encouraging results (Robert et al., 2010), providing some of the first evidence for the

feasibility of direct targeting of a selectively generated cryptic collagen element for treating malignant human tumors.

8 | CONCLUSIONS

Despite significant progress in the development of new clinical strategies to treat malignant tumors, including the recent successful use of immune checkpoint inhibitors, these approaches have shown activity in only a limited number of subjects and are often associated with the development of resistance mechanisms that inevitably limit their overall therapeutic benefit. Thus, it is clear that a more in-depth cellular and molecular understanding of the mechanisms governing tumor growth and metastasis is urgently needed to develop more effective treatments with more durable responses in a larger number of patients. Among some of the potential therapeutic advantages of direct targeting of cryptic collagen epitopes for the treatment of malignant tumors include the relatively selective generation of certain cryptic collagen epitopes within the tumor microenvironment, the possible impact of targeting the cryptic epitopes on the functions of both the tumor cells as well as multiple stromal cells that contribute to the disease progression, and finally, blocking important protumorigenic signaling cascades by targeting the ligand rather than the cell surface receptor, which would limit the impact of this therapeutic strategy to those tissues that have the appropriate cell surface receptors capable of responding to the cryptic collagen epitopes. These advantages would need to be considered within the context that certain cryptic collagen epitopes may also play roles in normal physiological processes, which might be disrupted by using this strategy.

In conclusion, direct targeting of cryptic signaling hubs that govern protumorigenic signaling pathways in both tumor cells as well as the wide array of stromal cells might allow for enhanced antitumor activity and potentially also provide a clinically useful strategy to increase the efficacy of current anticancer drugs. Due to the restricted exposure of cryptic collagen signaling hubs, using anti-cryptic collagen epitope antibodies or peptides alone or conjugating these reagents with cytotoxic drugs may provide an effective new method to localize clinically useful therapeutic payloads to multiple types of cancers. In fact, studies have documented that targeting the ECM of tumors with drug conjugated reagents may assist in localizing their therapeutic activity to the tumor microenvironment (Liang et al., 2015) and in some cases enhance the therapeutic activity of reagents such as immune checkpoint inhibitors (Ishihara et al., 2017). These and other studies are opening up the possibility of engineering a new generation of precision-guided therapeutics based on targeting unique cryptic ECM elements.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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The XL313 Cryptic Collagen Epitope Regulates Ovarian Tumor Growth in YAP Associated Mechanism

#6206

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Abstract

Structural changes in extra cellular matrix (ECM) can cause the exposure of cryptic regulatory sites that play important roles during angiogenesis, inflammation associated tumor growth, and tumor metastasis. Previously we discovered that a unique cryptic regulatory site, RGDKE containing XL313 cryptic collagen epitope, is generated during tumor development, and found that XL313 epitope promotes angiogenesis and inflammation in vivo. Here we provide first evidence that selectively blocking of cellular interaction between XL313 epitope and α V β 3 integrin significantly inhibits ovarian tumor growth in vivo. To understand the cellular mechanism(s) through which antagonists of the XL313 epitope inhibit these pathological processes, we determined interaction between ovarian cancer cell and the XL313 epitope together with potential intracellular downstream signaling. Here we show that the XL313 epitope binds to α V β 3 integrin receptor to promote ovarian cancer cell adhesion, and selectively targeting the XL313 epitope interrupts such binding. Importantly, selectively targeting the XL313 epitope significantly reduced nuclear accumulation of the transcriptional co-activator YAP through increasing YAP phosphorylation at serine127. Interestingly, specific targeting of the XL313 epitope also enhanced LAT1 phosphorylation, which is a key molecule for hippo pathway. Taken together, our data provide new insight for understanding the role of the XL313 cryptic collagen epitope in ovarian tumor growth, and suggest selectively targeting the XL313 epitope-integrin-signaling can be a novel strategy to inhibit ovarian cancer progression.

Results

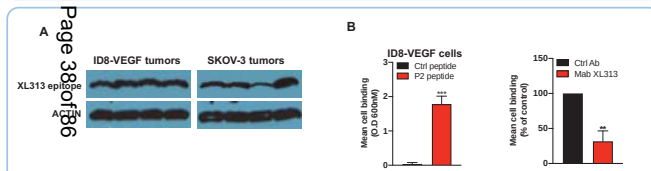


Figure 1. The RGDKE containing XL313 cryptic collagen epitope is expressed within the ID8-VEGF and SKOV-3 tumors, and it interacts with ovarian carcinoma cells. **A)** Expression of RGDKE containing XL313 epitope in ID8-VEGF and SKOV-3 ovarian tumors. **B)** Quantification of relative adhesion of ID8-VEGF cells to control peptide (CP) and RGDKE P2 peptide (Left). Quantification of relative adhesion of SKOV-3 cells to RGDKE P2 peptide in the presence of control or XL313 antibody (Right). **C)** Quantification of relative adhesion of SKOV-3 cells to RGDKE P2 peptide (Left). Quantification of relative adhesion of SKOV-3 cells to RGDKE P2 peptide with control antibody or XL313 antibody.

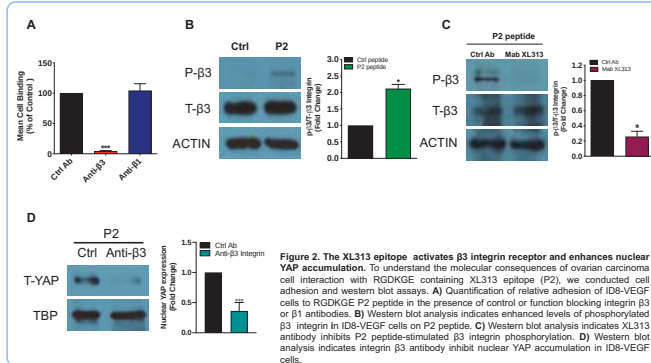


Figure 2. The XL313 epitope activates β 3 integrin receptor and enhances nuclear YAP accumulation. To understand the molecular consequences of ovarian carcinoma cell interaction with RGDKE containing XL313 epitope (P2), we conducted cell adhesion and western blot assays. **A)** Quantification of relative adhesion of ID8-VEGF cells to RGDKE P2 peptide in the presence of control or function blocking integrin β 3 or β 1 antibodies. **B)** Western blot analysis indicates enhanced levels of phosphorylated β 3 integrin in ID8-VEGF cells on P2 peptide. **C)** Western blot analysis indicates XL313 antibody inhibits P2 peptide-stimulated β 3 integrin phosphorylation. **D)** Western blot analysis indicates integrin β 3 antibody inhibit nuclear YAP accumulation in ID8-VEGF cells.

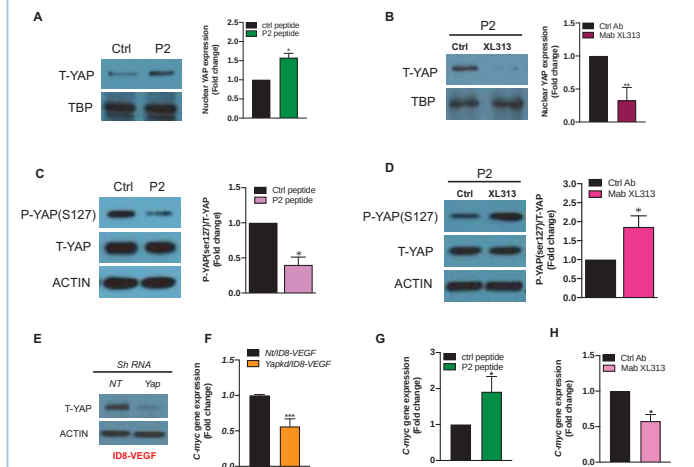


Figure 3. XL313 epitope regulates nuclear YAP accumulation, YAP phosphorylation, and YAP target gene *c-Myc* expression. Given that RGDKE containing XL313 epitope can activate integrin β 3 receptor and YAP activation is regulated by integrin β 3, we further examined the effect P2 peptide and its ability to alter nuclear accumulation of YAP in ovarian cancer cells. **A)** Western blot analysis indicates enhanced level of nuclear YAP in P2 peptide-stimulated ID8-VEGF cells as compared with control peptide. **B)** Western blot analysis indicates reduced levels of P2 peptide-stimulated nuclear YAP in ID8-VEGF cells treated with XL313 antibody. **C)** Western blot analysis indicates reduced levels of YAP phosphorylation in P2 peptide-stimulated ID8-VEGF cells as compared with control peptide. **D)** Western blot analysis indicates enhanced level of YAP phosphorylation in P2 peptide-stimulated ID8-VEGF cells treated with XL313 antibody. Given the role of YAP in regulating pro-tumorigenic genes expression, we examined whether YAP may play a role in regulating the expression of *c-Myc* by knocking down YAP in ID8-VEGF cells. **E)** Western blot analysis indicates YAP knock down is successful. **F)** qRT-PCR data indicates reduced levels of *c-Myc* gene expression in P2 peptide-stimulated ID8-VEGF cells treated with XL313 antibody. **G)** qRT-PCR data indicates enhanced levels of *c-Myc* expression in P2 peptide-stimulated ID8-VEGF cells as compared with control peptide. **H)** qRT-PCR data indicates reduced level of *c-Myc* expression in YAP knock down ID8-VEGF cells.

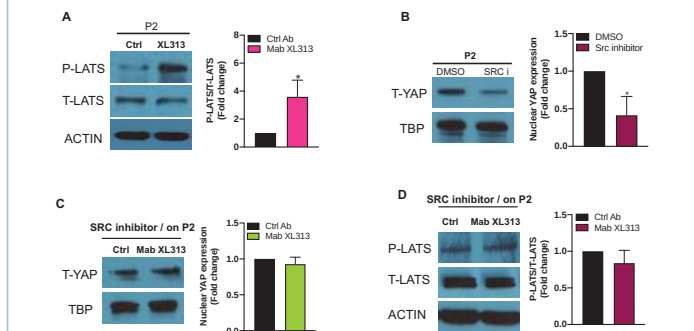


Figure 4. The ability of the Mab XL313 to regulate LAT1 phosphorylation and nuclear YAP accumulation is Src-dependent mechanism. Since the RGDKE containing XL313 epitope regulates nuclear accumulation of YAP in ovarian cancer cells and the hippo pathway effector kinase LAT1 is known to phosphorylate YAP leading to inactivation and reduced nuclear accumulation, we examined the level of phosphorylated LAT1 in P2 peptide-stimulated ID8-VEGF cells in the presence of Mab XL313 or a non specific control antibody. **A)** Western blot analysis indicates enhanced levels of phosphorylated LAT1 by blocking cellular interactions with P2 peptide. **B)** Western blot analysis indicates reduced level of nuclear YAP by Src inhibitor. **C)** Western blot analysis indicates that the ability of Mab XL313 to alter nuclear accumulation of YAP depends on Src. **D)** Western blot analysis indicates ability of Mab XL313 to alter phosphorylation of LAT1 depends on Src.

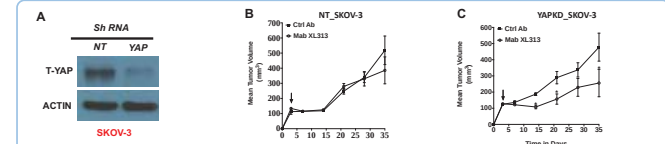
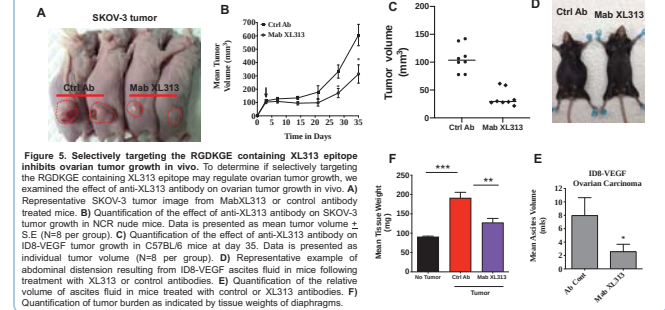
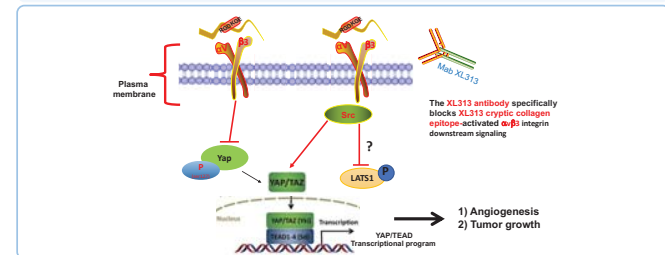


Figure 6. Inhibition of ovarian tumor growth by selectively targeting the RGDKE containing XL313 epitope depends on YAP. Studies indicate that the RGDKE containing XL313 epitope can bind to integrin β 3 and stimulate a signaling cascade leading to enhanced nuclear accumulation of YAP. Given these observations, we are wondering whether the anti-tumor activity of Mab depends on YAP knock down the YAP gene in SKOV-3 cells and examined the anti-tumor activity of Mab XL313 on the growth of either non-targeting or YAP knock down SKOV-3 cells. **A)** Western blot analysis indicates YAP knock down is successful. **B)** Quantification of the effect of anti-XL313 antibody on non-targeting SKOV-3 (NT-SKOV-3) tumor growth in mice. Data bars indicate mean tumor volume $\pm$ S.E. (N=8-10 per group). **C)** Quantification of the effect of anti-XL313 antibody on YAP knock down SKOV-3 (YAPKD-SKOV-3) tumor growth in mice. Data bars indicate mean tumor volume $\pm$ S.E. (N=8-10 per group).

Summary



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An RGDKGE containing cryptic collagen fragment regulates phosphorylation of LATS1 and controls ovarian tumor growth by a YAP-dependent mechanism.

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Running Title: Collagen fragment regulates ovarian tumors

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Abstract.

The growth and spread of malignant tumors such as ovarian carcinomas are governed in part by complex interconnected-signaling cascades occurring between stromal and tumor cells. These reciprocal cross-talk signaling networks operating within the local tissue microenvironment may enhance malignant tumor progression. Understanding how novel bioactive molecules generated within the tumor microenvironment regulate signaling pathways in distinct cellular compartments is critical for the development of more effective treatment paradigms. Here, we provide evidence that blocking cellular interactions with an RGDKGE-containing collagen peptide that selectively binds integrin $\beta 3$ on ovarian tumor cells, enhances the phosphorylation of the hippo effector kinase LATS1, and reduces nuclear accumulation of YAP and its target gene c-Myc. Selectively targeting this RGDKGE-containing collagen fragment inhibited ovarian tumor growth and the development of ascites fluid in vivo. These findings suggest that this bioactive collagen fragment may represent a previously unknown regulator of the hippo effector kinase LATS1 and regulates ovarian tumor growth by a YAP dependent mechanism. Taken together, these data not only provide new mechanistic insight into how a unique collagen fragment may regulate ovarian cancer, but in addition may help provide a potentially useful new alternative strategy to control ovarian tumor progression based on selectively disrupting a previously unappreciated signaling cascade.

Introduction

It is well established that the stromal microenvironment, which is composed of a variety of diverse cell types, secreted factors and a complex interconnected network of extracellular matrix (ECM) molecules can exert profound effects on tumors (1-3). Cellular interactions with specific ECM components such as collagen play central roles in controlling many pro-tumorigenic signaling programs that govern cellular proliferation, survival, migration and invasion. Dysregulation of these processes underlies many of the altered phenotypic characteristics observed in malignant tumors (4-6). Our laboratory has been focused on developing a more detailed cellular and molecular understanding of how both stromal and tumor cells utilize signaling cascades that are uniquely stimulated by cellular interactions with cryptic or hidden regions within ECM proteins.

Our previous studies have identified several matrix-immobilized cryptic integrin binding sites within ECM molecules that actively regulate angiogenesis, tumor growth and metastasis (7-9). A variety of studies suggest that in addition to structurally altered ECM proteins that remain immobilized within the insoluble matrix, bioactive fragments of ECM proteins can also be released in a soluble form (10-12). Elevated levels of some of these peptides have been shown to correlate with a more aggressive tumor phenotype in human subjects (12). In fact, new studies have now shown that elevated levels of a defined subsets of soluble collagen fragments detected in a cohort of cancer patients correlated with resistance to anti-CTLA-4 immune checkpoint therapy (13). These and many other studies are consistent with the possibility that a subset of soluble ECM fragments may not only represent clinically useful biomarkers for studying disease progression and resistance to therapy, but may also play fundamental roles in controlling angiogenesis, inflammation and tumor growth. In this regard, we previously identified an

endogenously expressed soluble collagen fragment generated by a subset of M2-like macrophages (11). This soluble RGDKGE-containing collagen fragment stimulated angiogenesis and inflammation in vivo (11). These observations were surprising given that many studies have shown that RGD-containing peptides often exhibit the opposite effect, and inhibit angiogenesis and tumor growth (14,15). This unexpected pro-angiogenic activity appeared to depend in part on the unique flanking sequences surrounding the core RGD motif, as other RGD-containing collagen peptides lacking this motif failed to significantly promote angiogenesis or inflammation in this model (11).

While this RGDKGE-containing collagen fragment selectively bound to vascular endothelial cells and stimulated angiogenesis that was dependent in part on the transcriptional coactivator Yes Associated Protein (YAP), it is not known whether this collagen fragment is generated in malignant tumors or whether it plays a functional role in tumor growth in vivo. While studies have implicated YAP in promoting the growth of different tumor types including ovarian carcinomas (16,17), other studies have suggested that YAP signaling might exhibit tumor suppressive activity depending on the specific cell type and tissue microenvironment (18,19). In fact, recent studies suggest that YAP activity in peritumoral hepatocytes may suppress the growth of liver tumors (19). Thus, a more in-depth molecular understanding of how YAP activity is controlled in context dependent pathological processes, may allow for the development of innovative new approaches to selectively regulate YAP within distinct tissue microenvironments.

A wide array of molecular regulators and mechanisms have been identified that govern the activation and nuclear accumulation of YAP. Among the mechanisms by which nuclear YAP accumulation is thought to be controlled include the canonical hippo signaling cascade (20,21). While the ability of the core kinases within the hippo signaling cascade to control YAP activity

is well documented, little is known concerning the possibility that soluble collagen derived fragments may regulate the hippo pathway or whether targeting these extracellular regulators may be exploited to selectively control YAP activity during tumor growth in vivo. Here, we provide evidence that blocking a soluble RGDKGE-containing collagen fragment can regulate the large tumor suppressor kinase-1 (LATS1), a core effector molecule within the hippo pathway. We show that ovarian cancer cell interactions with a RGDKGE collagen peptide activates $\beta 3$ integrin and reduces the level of inhibitory phosphorylation of YAP and enhances its nuclear accumulation. Moreover, selectively blocking cellular interactions with the RGDKGE collagen peptide, enhanced the phosphorylation of LATS1 and YAP, reduces nuclear accumulation of YAP and also reduced the levels of the YAP target gene c-Myc. Importantly, selectively blocking cellular interactions with the RGDKGE-containing collagen fragment inhibited the development of ascites fluid and ovarian tumors growing in vivo. These findings indicate that a soluble RGDKGE-containing collagen fragment may represent a previously unknown regulator of the hippo effector kinase LATS1 that plays a role in controlling ovarian tumor growth. These data not only provide new mechanistic insight into how an endogenously generated bioactive collagen fragment may regulate ovarian cancer, but also provide an alternative strategy for the development of new therapeutic approaches to control ovarian tumor progression.

Materials and Methods

Cells and cell culture

Murine ID8-VEGF ovarian carcinoma cells (22) were obtained from Dr. Philip Greenberg (University of Washington School of Medicine, Seattle WA). ID8-VEGF cells were cultured in

DMEM in the presence of 4% FBS, 1.0% Pen-Strep, 1.0% Sodium pyruvate, and 1% of insulin-transferrin-sodium selenite media supplements (ITS). SKOV-3 ovarian carcinoma cells were obtained from ATCC (Manassas, VA) and cultured in RPMI1640 medium in the presence of 5% FBS, 1% Pen-strep and 1% sodium pyruvate.

Animals

C57BL/6J female mice (6-8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME). NCRNU-F female mice (6-8 weeks old) were obtained from Taconic Biosciences (Germantown, NY). Mice were housed in Maine Medical Center Research Institute pathogen-free air barrier facility, and all animal handling and procedures were approved by the Maine Medical Center Institutional Animal Care and Use Committee.

Reagents, chemicals and antibodies

Bovine serum albumin (BSA), insulin-transferrin-sodium selenite media supplements (ITS) and crystal violet were obtained from Sigma (St Louis, MO). Anti-YAP1, anti-phospho-YAP (Ser127), anti-LATS1, anti-phospho-LATS (Thr 1079), anti-SRC, anti-phospho-SRC (Y416), anti-Phospho-MST1-Thr-183, anti-integrin β 3, and anti-TATA-binding protein (TBP) antibodies were all obtained from Cell Signaling (Danvers, MA). Anti-phospho-integrin β 3 (Ser747) and anti- β -Actin were obtained from the Santa Cruz (Dallas, TX). Anti-MST1 antibody was obtained from Novus Biologicals (Centennial, CO). Anti-CD-31 antibody was obtained from Abcam (Cambridge, MA). Mab XL313 and non-specific control antibody was obtained from Bio X Cell (Lebanon, NH). P2 peptide (QGPGGRDKGE) and control peptide CP (CQGPGEAAGG) were from QED Bioscience (San Diego, CA). Alexa 594 labeled secondary

antibody was from Invitrogen (Carlsbad, CA). The SRC inhibitor Dasatinib was from Selleckchem (San Diego, CA).

Western blot analysis

ID8-VEGF or SKOV-3 cells from sub-confluent culture were serum starved in suspension for 1hr at 37°C, and seeded on control peptide (CP) or RGDKGE-containing peptide (P2) coated plates in the presence or absence of Mab XL313 or control antibody for 5 or 15 minutes under the defined experimental conditions and then lysed in RIPA lysis buffer with 1X protease inhibitor cocktail, 2mM of PMSF, 1mM of sodium orthovanadate (Santa Cruz, CA). Equal amounts of cell lysate were separated by SDS PAGE. Membranes were probed with the indicated antibodies. Western blots were performed at least 3 to 4 times and visualized by chemiluminescence detection. Quantification of the mean relative changes in proteins was carried out using image J software based on pixel intensity (9).

Nuclear protein extraction

Sub-confluent ID8-VEGF cells were washed and serum starved for 1hr at 37°C and cells were then seeded on 6-well plates coated (100µg/ml) with control peptide (CP) or RGDKGE-containing peptide (P2). To examine the effects of blocking cellular interactions with the RGDKGE-containing collagen peptide P2 on nuclear accumulation of YAP, cells were seeded on P2 peptide (100µg/ml)-coated 6-well plates that were pre-blocked with 100µg/ml of Mab XL313, anti-integrin $\beta 3$ antibody or non-specific control antibody for 30 minutes. To examine the effects of Src on YAP, ID8-VEGF cells from sub-confluent culture were washed and serum starved for 30 minutes at 37°C and cells were pre-treated with Src inhibitor Dasatinib (1µM) or

DMSO for 30 minutes. Next, cells were seeded on P2 peptide coated 6-well plates in presence or absence of Mab XL313 antibody or control antibody. After incubating for 1hr, adherent and non-adherent cells were harvested. The nuclear and cytoplasmic proteins were extracted using NE-PER nuclear and cytoplasmic extraction reagents from Thermo Scientific (Waltham, MA) according to the manufacture instructions. The nuclear extractions were examined by western blotting analysis with anti-YAP or anti-TBP (TATA binding protein) antibody, and the cytoplasmic extraction was examined by western blotting analysis with total and phospho-YAP (Ser-127), total and phospho-LATS1 (Ser-1079), or total and phospho-MST1 (Thr-183) antibodies.

RNA isolation, cDNA synthesis and quantitative PCR

Sub-confluent ID8-VEGF cells were washed and serum starved for 1hr at 37°C, and cells were seeded on 6-well plates coated with control peptide CP or RGDKGE-containing peptide P2 (100 µg/ml). To examine the effect of blocking cellular interactions with the RGDKGE-containing peptide P2 had on c-Myc, cells were washed and serum starved for 30 minutes at 37°C and pre-blocked with 100 µg/ml of control or Mab XL313 antibodies. Next cells were seeded on P2 peptide (100 µg/ml)-coated 6-well plates. After 1hr incubation, cells were lysed using RNA isolation lysis buffer, and RNA were isolated by using RNase plus mini kit (Qiagen, Carol Stream, IL) from the lysate followed by manufactured protocol. Total RNA (1.0 µg) was synthesized with iScript cDNA synthesis kit from Bio-Rad (Hercules, CA). Equivalent amount of cDNA was used for qRT-PCR, and the murine qRT-PCR primers used for mouse *c-Myc* were (Forward: 5'-CAGAGGAGGAACGAGCTG-3'; Reverse: 5'-TTATGCACCAGAGTTTCGAAGCTGTTCGT-3') and for the housekeeping gene mouse *b2m*,

the primers were (Forward: 5'-CTGACCGGCCTGTATGCTAT-3'; Reverse: 5'-CCGTTCTTCCAGCATTTGGAT-3'). qRT-PCR assays were repeated 5 times.

Analysis and quantification of tumor angiogenesis

Frozen sections from individual tumors (N=5 per condition) were fixed in 50% methanol 50% acetone, and blocked with 2.0% BSA for 1hr at room temperature. For quantification of angiogenesis, frozen sections of tumors from SKOV-3 ovarian tumors were stained with anti-CD31 (1:300) overnight, followed by incubation with Alexa 594 labeled secondary antibody. Tumor vessel counts were carried out from five 200X microscopic fields from each of 5 independent tumors from each condition.

Preparation of whole tumor lysates

SKOV-3 and ID8-VEGF ovarian tumors (N=4 per tumor type) were harvested from nude mice or C57BL/6 mice at day-14 or day-7 respectively. Individual tumors were snap frozen on dry ice and ground in a cold mortar. Ground up tissues from individual tumors were next mixed with RIPA lysis buffer and whole tissue lysates were generated.

Cell binding assays

Forty-eight well non-tissue culture plates were coated with 100 µg/ml of control peptide (CP) or RGDKGE-containing peptide (P2). Tumor cells were suspended in adhesion buffer (RPMI containing 1mM MgCl₂, 0.2mM MnCl₂ and 0.5% BSA) and 1x10⁵ cells were added to the wells in the presence or absence of 100 µg/ml of Mab XL313, anti-integrin β3 antibody, anti-integrin β1 or non-specific normal mouse IgG control antibody and allowed to bind for 30-45 minutes at

37°C. Non-attached cells were removed and attached cells were stained with crystal violet (7). Cell binding was quantified by measuring the optical density of eluted dye (7). Cell binding assays were performed at least 3 times with triplicate wells per condition.

Viral vectors and transduction

Lentiviral vectors (pLKO.1 based) encoding short hairpin RNAs (shRNAs) specific to YAP or a control non-targeting construct developed by the RNAi Consortium were obtained from GE Healthcare life science (Lafayette, CO). The effective targeting sequence of YAP gene was 5'-TTCTTTATCTAGCTTGGTGGC -3'. Lentivirus was packaged in the recombinant viral vector core facility at Maine Medical Center Research Institute. Cells transduced with the shRNA viruses were selected in growth medium containing 2.5 µg/ml puromycin (Invitrogen) to establish cell pools stably expressing shRNA.

Tumor growth assays

Tumor growth assays were performed as previously described (9). Briefly, mice were injected subcutaneously with either 10×10^6 ID8-VEGF cells (N= 8-10 C57BL/6 mice per condition), or 3×10^6 SKOV-3 cells, non-target SKOV-3 cells or YAP knock-down SKOV-3 cells (N=8-10 NCRNU-F mice per condition). Tumors were allowed to form palpable tumors for 3 days, then the mice were injected (i.p.) with Mab XL313 (25 - 250 µg/mouse) or nonspecific control antibody (250 µg/mouse) 3 times per week for either 42 days for ID8-VEGF tumor model, or 35 days for the SKOV-3 tumor model. Tumor size was measured with caliper and the tumor volume was calculated using the formula $V = L^2 \times W/2$, where V= volume, L= length, W=width. For

ascites tumor assays, 10×10^6 ID8-VEGF cells were injected (i.p.) in C57BL/6J female mice (N=8). Four weeks later, mice were i.p. injected with Mab XL313 or control antibody (100 µg/mouse) twice a week. At the end of the 13 weeks treatment period, ascites fluid was harvested and the volume was quantified. To estimate tumor burden within the diaphragm, mouse diaphragms were harvested and wet tissue weights were determined (N=5 per group).

Statistical analysis

Statistical analysis was performed using the Prism/Graph Pad statistical program for Macintosh computers. Data were analyzed for statistical significance using student t-test. P values < 0.05 were considered significant.

Results

Detection of an RGDKGE-containing collagen fragment in ovarian tumors and interaction with ovarian carcinoma cells. Our previous studies identified an endogenously generated cryptic collagen fragment containing the amino acid sequence RGDKGE that could be detected as an approximately 16Kd soluble fragment in serum free conditioned medium from M2-like macrophages (11). Given these studies, we sought to determine whether this collagen fragment might be generated during tumor growth. To identify potentially relevant tumor models with which to study this collagen fragment, we examined whole tissue lysates of SKOV-3 and ID8 - VEGF ovarian tumors growing in mice for the generation of the 16Kd RGDKGE-containing collagen fragment. As shown in figure 1A and B, the 16Kd RGDKGE-containing collagen fragment was detected within whole tumor lysates from both SKOV-3 and ID8-VEGF tumors

growing in vivo. These data suggest that ovarian tumors may represent a biologically relevant tumor type to study the possible impact this collagen fragment might have on tumor growth.

While our previous studies indicated that vascular endothelial cells could directly bind to the RGDKGE collagen fragment (11), it is not known whether ovarian tumor cells themselves also have this binding ability. Therefore, in order to further study potential roles of this collagen fragment might have in ovarian cancer, we assessed whether ovarian carcinoma cells have the capacity to directly interact with a synthetic RGDKGE-containing collagen peptide we have called P2 (11). As shown in figure 1C and D, SKOV-3 and ID8-VEGF tumor cells readily bound to the P2 collagen peptide, while exhibiting minimal if any binding with the non-specific control peptide CP. Importantly, Mab XL313 specifically directed to the RGDKGE-containing peptide P2, inhibited cellular binding to P2 as compared to a non-specific control antibody (Figure 1E and F). These data indicate that SKOV-3 and ID8-VEGF tumor cells can interact with the RGDKGE collagen fragment and may represent useful tumor models with which to study its possible functions.

The RGDKGE-containing collagen fragment activates β 3 integrin and stimulates enhanced nuclear accumulation of YAP. To determine potential receptors capable of mediating cellular interactions with the RGDKGE containing collagen peptide P2, cell binding assays were carried out in the presence of function blocking anti-integrin antibodies. As shown in figure 2A and B, function blocking antibodies directed to β 3 integrin significantly inhibited binding of both SKOV-3 and ID8-VEGF tumor cells to the P2 collagen peptide by over 80%, while a function blocking antibody directed to β 1 integrins had no significant inhibitory activity (Figure 2A and

B). These data indicate that $\beta 3$ integrin can serve as a cell surface receptor for the P2 collagen peptide in these ovarian tumor cells.

Next, we examined whether cellular interactions with the P2 collagen peptide was sufficient to initiate activation of $\beta 3$ integrin as indicated by phosphorylation of $\beta 3$ at tyrosine 747, which is known to facilitate down-stream signaling events (23). To study this possibility, we focused on ID8-VEGF cells given their relatively high levels of $\beta 3$ integrin (Supplemental Figure 1A and B). To this end, ID8-VEGF cells were stimulated by allowing tumor cells to bind to the P2 collagen peptide or a non-specific control peptide CP and cell lysates were prepared. As shown in figure 2C and D, tumor cell binding to the P2 collagen peptide significantly enhanced tyrosine phosphorylation of $\beta 3$ integrin by approximately 50%. In contrast, blocking binding of P2 to the ovarian tumor cells with Mab XL313, inhibited $\beta 3$ integrin phosphorylation (Figure 2E and F). These data indicate that the P2 collagen peptide can directly regulate $\beta 3$ -integrin activation, while Mab XL313 selectively inhibit this $\beta 3$ -integrin activation.

Interestingly, our previous studies suggested that the RGDKGE collagen peptide could stimulate enhanced nuclear accumulation of YAP in endothelial cells (11). Given the ability of the RGDKGE collagen fragment to bind and activate $\beta 3$ -integrin in ovarian carcinoma cells, we sought to determine whether $\beta 3$ -integrin receptor may also play a role in controlling P2 peptide stimulated nuclear YAP accumulation in tumor cells. In this regard, we stimulated ID8-VEGF cells with P2 collagen peptide and examined the effects of a function blocking anti- $\beta 3$ integrin antibody had on nuclear YAP accumulation. As shown in figure 2G and H, blocking P2 peptide cellular interactions with ID8-VEGF tumor cells by incubation with anti- $\beta 3$ integrin antibody, significantly reduced the relative levels of nuclear YAP. Taken together these data provide the

first evidence that blocking $\beta 3$ integrin-mediated interactions with the RGDKGE-containing collagen peptide P2 inhibits nuclear YAP accumulation in these ovarian carcinoma cells.

The RGDKGE-containing collagen fragment regulates YAP phosphorylation in ovarian tumor cells. Previous studies have implicated integrin signaling in regulating the activity and nuclear accumulation of YAP in different cell types (11,24-26). However, YAP can be controlled by diverse mechanisms, and depending on the particular cell type, may have opposing functions on tumor growth (16,18,19). Therefore, we examined in more detail the effects of stimulation of ovarian tumor cells with the RGDKGE collagen peptide P2 and its ability to alter nuclear accumulation of YAP. As shown in figure 3A left and right panels, ID8-VEGF tumor cell binding to P2 resulted in significantly enhanced levels of nuclear localized YAP, while treatment with Mab XL313, but not control antibody inhibited nuclear YAP levels (Figure 3B left and right panels). Nuclear localized YAP can interact with transcription factors such as members of TEAD family to regulate pro-tumorigenic gene expression programs that contribute to tumor growth and metastasis (27,28). Because YAP regulates the expression of pro-tumorigenic target genes that are thought to play a role in ovarian cancer such as c-Myc (27-30), we examined whether YAP may play a role in regulating the expression of c-Myc in ID8-VEGF tumor cells. To facilitate these studies we knocked down YAP in ID8-VEGF cells (Supplemental Figure 1C) using a YAP specific shRNA, and the relative levels of c-Myc mRNA was examined. As shown in figure 3C, knocking down YAP significantly reduced the levels of c-Myc as compared to control transfected cells, confirming the relevance of YAP in regulating c-Myc in these ovarian tumor cells. Given our findings that cellular interactions with the RGDKGE-containing collagen peptide P2 regulates YAP, we next examined the effects of P2 stimulation on the relative levels

of c-Myc mRNA by qRT-PCR. As expected, tumor cell interaction with the RGDKGE collagen fragment significantly enhanced c-Myc (Figure 3D), while blocking cellular engagement with the RGDKGE collagen fragment reduced c-Myc mRNA levels (Figure 3E). These data are consistent with the ability of RGDKGE collagen fragment to modulate the expression of a known YAP-target gene.

The differential localization of YAP between the cytoplasm and the nucleus where it can function as a transcriptional co-activator regulating gene expression programs is thought to be controlled by multiple mechanisms including hippo-dependent and hippo-independent signaling events (16,20,21). To uncover possible mechanisms that might contribute to the ability of this collagen peptide to enhance YAP nuclear localization, we examined YAP phosphorylation, since the phosphorylation of YAP on serine-127 has been shown to play a role in limiting the relative levels of active YAP within the nucleus (20,21). Interestingly, ID8-VEGF cell interactions with P2 collagen peptide significantly inhibited the levels of YAP serine 127 phosphorylation (Figure 3F and G), while blocking cellular interactions with P2 enhanced YAP phosphorylation (Figure 3H and I). Collectively, these findings suggest that cellular interactions with the RGDKGE collagen fragment can enhance nuclear accumulation of YAP in ovarian carcinoma cells and that this differential accumulation of nuclear YAP was associated with alterations in YAP phosphorylation and expression of the YAP target gene c-Myc.

The RGDKGE-containing collagen fragment regulates the hippo pathway effector kinase LATS1 by a Src-dependent mechanism. As mentioned above, the nuclear accumulation of YAP is thought to be governed by multiple mechanisms (16,20,21). The large tumor suppressor kinase-1 (LATS1) is a well-established effector molecule of the hippo signaling cascade known

to phosphorylate YAP leading to inactivation and reduced nuclear accumulation (20,21,31,32). In this regard, we examined the levels of phosphorylated LATS1 in ID8-VEGF tumor cells following stimulation with P2 collagen peptide in the presence of Mab XL313 or a non-specific control antibody. As shown in figure 4A and B, the levels of phosphorylated LATS1 on Ser-1079 was significantly enhanced by over 3-fold following blocking cellular interactions with P2 collagen peptide. Interestingly, blocking cellular interactions with P2 had no detectable effect on the relative levels of activated hippo kinase MST1 (Supplemental Figure 1D). These data are consistent with the ability of Mab XL313 to inhibit nuclear accumulation of YAP that is associated with the regulation of LATS1, but independent from changes in activation of MST1.

Given that both LATS1 and YAP have been suggested to be controlled in part by Src family kinases (33-35), and that Src is a major down-stream effector molecule that can be regulated by integrin signaling, we examined whether Src plays a role in the ability of the P2 collagen fragment to control YAP nuclear accumulation. To this end, we first examined the relative levels of P2-stimulated nuclear YAP in tumor cells in which Src was inhibited. Briefly, ID8-VEGF cells were first pre-incubated with the Src inhibitor Dasatinib to block Src activity (Supplemental Figure 1E) and these cells were then stimulated with P2 and the relative levels of nuclear YAP was quantified. As shown in figure 4C and D, inhibiting Src reduced the relative levels of P2 peptide stimulated nuclear YAP as compared to control. Moreover, inhibiting Src also prevented the ability of Mab XL313 to alter nuclear accumulation of YAP, as Mab XL313 failed to significantly reduce the levels of nuclear YAP in Src inhibited cells (Figure 4E and F).

Given that blocking cellular interactions with the P2 collagen peptide significantly enhanced phosphorylated LATS1, we next examined the effects of Src inhibition has on the ability of Mab XL313 to control LATS1 phosphorylation. As expected, blocking Src inhibited the ability of

Mab XL313 to regulate LATS1 phosphorylation (Figure 4G and H). Taken together, these findings suggest that the ability of the RGDKGE collagen fragment to regulate the hippo effector kinase LATS1 and its down-stream target YAP in these tumor cells depends on Src.

Selective targeting of the RGDKGE containing collagen fragment inhibits ovarian tumor growth. The RGDKGE collagen fragment was previously shown to play a role in angiogenesis and inflammation (11). However, it is not known whether this collagen fragment may impact tumor growth in vivo. Given our current observations, we sought to examine whether selective targeting this collagen fragment might regulate ovarian tumor growth. To examine this possibility, we first assessed the dose dependent effects of Mab XL313 on ovarian tumors growing in mice. Human SKOV-3 ovarian tumor cells were injected subcutaneously. Once tumors reached a size of approximately 100mm³ mice were treated with Mab XL313 or non-specific control antibody three times per week at the doses indicated. As shown in figure 5A, treatment with Mab XL313 exhibited dose dependent inhibitory activity, and showed a significant inhibition of approximately 50% on day-35. Given our previous studies indicating that selectively targeting the RGDKGE collagen fragment could inhibit growth factor-induced angiogenesis in the chick embryo model (11), we examined SKOV-3 tumors for the relative levels of angiogenesis by quantifying the numbers of CD31-expressing tumor vessels. Tumors from mice treated with Mab XL313 resulted in significantly reduced levels of tumor vessels by approximately 35% (Figure 5B). To confirm the anti-tumor activity of Mab XL313 in a second ovarian tumor model, we examined the effects of Mab XL313 on ID8-VEGF tumor growth. Murine ID8-VEGF tumor cells were injected subcutaneously and beginning on day 3, mice were treated as before. While these subcutaneous ID8-VEGF tumors grew slowly and showed

evidence of regression irrespective of the treatment, the size of ID8-VEGF tumors from mice treated with Mab XL313 were significantly reduced by approximately 50% as compared to control (Figure 5C).

Ovarian tumors growing within the subcutaneous space may not represent an optimal model to study ovarian tumor growth due in part to the lack of a mesothelial cell layer covering tissues that are typically colonized within the peritoneal cavity where ovarian tumors typically grow (36,37). Therefore, we next examined the effects of targeting the RGDKGE collagen fragment might have on ID8-VEGF ascites fluid formation and tumor colonization of the diaphragm, which represents a common tissue location of ovarian tumors in mice and humans (36,37). To this end, mice were injected intraperitoneally with ID8-VEGF cells and allowed to grow for 4 weeks prior to initiating treatment. Beginning at 4 weeks, mice were either treated with non-specific control antibody or Mab XL313 two times per week. At the end of 13 weeks, mice were sacrificed and ascites fluid and diaphragms were examined. As shown in figure 5D, mice treated with control antibody formed ascites as indicated by extensive distention of the abdominal cavities, while mice treated with Mab XL313 exhibited less abdominal distension. Quantification of the relative volume of tumor ascites fluid indicated that mice treated with Mab XL313 had significantly reduced levels by over 60% (Figure 5E). Moreover, quantification of tumor burden as indicated by wet tissue weights of the diaphragms, indicated that mice treated with Mab XL313 exhibited an approximately 30% reduction in wet weight as compared to controls (Figure 5F). These novel findings suggest that the endogenously generated RGDKGE collagen fragment plays a role in ovarian tumor growth in vivo.

Mab XL313-mediated inhibition of ovarian tumor growth depends on altering YAP. The transcriptional co-activator YAP plays roles in angiogenesis, tumor growth and metastasis in multiple tumor models (16,24-28). However, other studies have suggested that YAP may have tumor suppressive functions, depending on the tissue location and cell type (18,19). Our current studies indicate that the RGDKGE-containing collagen fragment can bind to integrin $\beta 3$ and stimulate a signaling cascade leading to enhanced nuclear accumulation of YAP. Given these observations, we sought to determine whether the anti-tumor activity of Mab XL313 depends on altering YAP. To facilitate these studies, we knocked down expression of YAP in SKOV-3 cells using a YAP specific shRNA (Supplemental Figure 1F), and examined the anti-tumor activity of Mab XL313 on the growth of either control transfected SKOV-3 cells (NT-SKOV-3) or on SKOV-3 cells in which YAP was knocked down (YAP-KD-SKOV-3). Tumor cell variants were injected in mice, and beginning on day 3, mice were treated 3 times per week with 100 μ g of Mab XL313 or control antibody. As shown in figure 6A, Mab XL313 significantly inhibited the growth of control transfected (NT-SKOV-3) tumors by approximately 50% on day 35. In contrast, Mab XL313 failed to significantly inhibit the growth of SKOV-3 tumors (YAP-KD-SKOV-3) in which the levels of YAP had been reduced (Figure 6B). These data are consistent with notion that the anti-tumorigenic activity of Mab XL313 depends on inhibiting YAP. Collectively, our data are consistent with a working model in which ovarian tumor cell interactions with an endogenously generated RGDKGE-containing collagen fragment stimulates a $\beta 3$ -integrin signaling cascade that results in suppressing phosphorylation of the effector kinase LATS1, ultimately leading to enhanced nuclear accumulation of YAP (Figure 6C).

Discussion. While the ability of the local stromal microenvironment to control tumor growth has been appreciated for years, effectively translating this cellular and molecular insight into new therapeutic strategies have been slow to emerge, due in part to the complexity of the various functional elements that constitute the tumor microenvironment. Stromal cells known to influence tumor growth include endothelial cells and a variety of differentially polarized fibroblasts and immune cells (1-3). These diverse groups of cells not only modulate the behavior of solid tumors by secreting bioactive molecules that can alter the structural integrity of the ECM, but they also secrete many soluble factors that directly bind to nearby tumor cells and modulate diverse signaling pathways (1-6,37,38). In a reciprocal manner, stromal cells can also respond to soluble factors released from tumor cells, and these soluble cell-cell communication pathways help support tumor progression (1-6,37,38). This complex cross-talk occurring between different cell types and between cells and a variety of ECM components plays critical roles in regulating tumor growth and metastasis, as well as influencing how tumors respond to different cancer therapies (1-6,37,38). Identifying soluble bioactive molecules that have the capacity to alter the behavior of both stromal and tumor cells will likely provide important new insight into how cross-talk signaling mechanisms are coordinated, and may help with the design of more effective therapeutic strategies to manage malignant tumor progression.

We previously identified a 16Kd RGDKGE-containing collagen fragment which was actively secreted by M2-like macrophages and stimulated nuclear accumulation of YAP in endothelial cells (11). While it is well documented that YAP can differentially regulate a number of distinct gene expression programs in different cell types, the role of YAP in controlling biological processes such as organ size, stem cell functions, differentiation and tumor growth likely depend on how YAP activity is controlled in distinct cell populations within a given tissue

microenvironment (39-41). In fact, the precise biological impact of YAP as it relates to both normal physiological processes as well as diseases such as cancer has been shown to be context dependent (39-41). For example, YAP has been shown to control the size of organs such as the heart and liver (42-44), while loss of YAP had minimal effects or reduced the size of other histologically distinct organs such as the intestine and pancreas (45-47). Moreover, while reducing YAP activity inhibits the growth of tumors such as melanoma, and lung carcinomas (48,49), other studies suggested that YAP under specific circumstances may have tumor suppressive functions in breast and liver carcinomas (18, 19). These and other studies indicate the need for a more in-depth understanding of the different mechanisms by which YAP is regulated within distinct cell types.

Uncovering novel mechanisms by which YAP may be regulated under pathological conditions may allow for more optimized strategies to control YAP activity in the context of tumor growth. YAP activity can be regulated by diverse mechanisms including, cell adhesion, mechanical forces, stress signaling and soluble factors (16,20,21), some of which can be altered by the canonical hippo signaling cascade (16,20,21). In addition, YAP can also be regulated by hippo-pathway independent mechanisms such as those involved in altered actin polymerization (16,20,21). Given these facts, coupled with the known functions of YAP in controlling gene expression programs (27,28), defining strategies to selectively control YAP activity under pathological conditions will likely contribute to optimizing the therapeutic efficacy of targeting YAP.

While intact collagen within the insoluble ECM is thought to regulate ovarian carcinoma progression (36,50), little is known as to whether soluble bioactive collagen fragments regulate ovarian tumor growth in vivo. Here, we provide evidence for the first time that a unique

RGDKGE-containing collagen fragment can be detected in ovarian tumors growing in mice. Given our previous studies, we sought to determine whether it might also directly bind to ovarian carcinoma cells and regulate tumor growth. Our data indicate that SKOV-3 and ID8-VEGF ovarian tumor cells can bind to the soluble RGDKGE collagen fragment using $\beta 3$ integrin, as a function blocking antibody directed to this integrin, inhibited binding while a function blocking antibody directed to $\beta 1$ -containing integrins failed to demonstrate any effect. Ovarian tumor cell binding to the RGDKGE collagen peptide lead to enhanced phosphorylation and activation of $\beta 3$ integrin, and enhanced nuclear accumulation of YAP, which could be specifically inhibited by directly targeting the RGDKGE collagen peptide with Mab XL313. Importantly, the exact molecular mechanism by which Src controls the ability of the RGDKGE collagen fragment to regulate LATS1 and YAP is not completely understood, and work is currently underway to define this signaling pathway.

The precise role of $\beta 3$ integrin in controlling tumor growth is complex as studies have provided evidence for both pro-tumorigenic as well as tumor suppressive functions. For example, studies have shown correlations between high expression of $\beta 3$ integrin and prognosis in patients with melanoma and breast carcinoma (51,52). Other studies using animal models have shown that directly blocking $\beta 3$ integrin inhibits angiogenesis and tumor growth (14,15,53,54). In contrast, other work examining ovarian carcinomas have shown that high levels of $\beta 3$ integrin correlated with improved clinical outcome and that reducing $\beta 3$ integrin expression enhanced ovarian tumor growth (55). These seemingly opposing functions for $\beta 3$ integrin might be explained at least in part, by the fact that the final outcome of $\beta 3$ integrin signaling within a particular cell or tumor type depends in part, on the nature of the different ligands binding to this integrin, and the types of adaptor molecules that are assembled into signaling complexes that

facilitate transmission of extracellular cues. For example, it is well established that certain $\beta 3$ integrin binding ligands such as vitronectin, osteopontin and periostin can induce pro-tumorigenic signaling events (56-58). In contrast, other bioactive factors that bind to $\beta 3$ integrin such as tumstatin, canstatin and endostatin have been shown to inhibit tumor growth (59-61). Interestingly, over expression of $\beta 3$ integrin in some ovarian tumor cell lines resulted in reduced proliferation that was associated with a G2/M block in the cell cycle and altered phosphorylation of CD2 (55). In other studies, evidence suggest that unoccupied integrin $\alpha v\beta 3$ may result in signaling leading to a process termed integrin mediated cell death (IMD) (62). The ability of $\alpha v\beta 3$ to control distinct functions in different cell types such as immune cells may also impact the overall contribution of $\alpha v\beta 3$ to tumor growth. For example, $\alpha v\beta 3$ expressed in macrophages and dendritic cells has been shown to play roles in phagocytosis of tumor cells as well as promoting antigen presentation (63,64). In addition, $\alpha v\beta 3$ has also been shown to play a role in regulating T-cell receptor signaling and motility (65,66), all of which may depend on the molecular features of the different ligands that bind to this integrin. In fact, studies have shown that direct targeting of $\beta 3$ or reducing $\beta 3$ integrin in a subpopulation of immune cells result in the induction of immune suppression (63,64), thereby compromising the efficacy of this therapeutic strategy as it relates to tumor growth. Therefore, designing and evaluating alternative strategies to disrupt $\alpha v\beta 3$ -dependent signaling without directly targeting $\alpha v\beta 3$ itself may help in the development of more effective treatments.

Our findings and many other studies are consistent with the notion that the overall contribution of $\alpha v\beta 3$ to tumor growth depends on a complex and interconnected network of parameters that include the cell type that the integrin is functioning in, the molecular characteristics of the local $\alpha v\beta 3$ binding ligands, and the bioavailability and composition of the

down-stream signaling molecules required for the formation of intracellular signaling complexes that facilitate activation of distinct signaling cascades. Given this complexity, a therapeutic strategy of directly targeting all $\alpha v \beta 3$ in different cell type may not represent an optimal approach. As an alternatively strategy, we have been studying whether selective targeting of discrete pro-tumorigenic ligands of integrin $\alpha v \beta 3$, instead of directly targeting the integrin itself, may provide a clinically effective new approach to control pro-tumorigenic signaling events. This alternative strategy might allow for selective inhibition of pro-tumorigenic stimuli mediated through integrin $\alpha v \beta 3$, and at the same, time allow for the engagement of locally available tumor suppressing ligands to simulate inhibitory signals through $\alpha v \beta 3$ within distinct stromal and tumor cell populations, ultimately promoting a more robust therapeutic out come when used either alone or in combination with other cancer treatments. Taken together, a therapeutic strategy that focuses on selectively targeting a novel pro-tumorigenic bioactive ligand of $\alpha v \beta 3$ integrin rather the directly targeting the $\alpha v \beta 3$ receptor itself, may help selectively disrupt pro-tumorigenic signaling pathways operating within both tumor and stroma cells to allow for an effective treatment paradigm for managing ovarian cancer.

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Figure Legends

Figure 1 Detection of the RGDKGE-containing collagen fragment in ovarian tumors and its binding to tumor cells. **A:** Whole tumor lysates were prepared from individual human SKOV-3 tumors (N=4) grown in nude mice. Example of western blot analysis for the levels of the 16Kd RGDKGE-containing collagen fragment or loading control actin. **B:** Whole tumor lysates were prepared from individual mouse ID8-VEGF tumors (N=4) grown in C57BL/6 mice. Example of western blot analysis for the levels of the 16Kd RGDKGE-containing collagen fragment or loading control actin. **C:** Quantification of SKOV-3 cell binding to control peptide CP and collagen peptide P2. **D:** Quantification of ID8-VEGF cell binding to control peptide CP and collagen peptide P2. **E:** Quantification of SKOV-3 cell binding to collagen peptide P2 in the presence of non-specific control antibody (Ab Cont) or anti-RGDKGE antibody (Anti-XL313 Mab). **F:** Quantification of ID8-VEGF cell binding to collagen peptide P2 in the presence of non-specific control antibody (Ab Cont) or anti-RGDKGE antibody (Anti-XL313 Mab). Data bars represent means $\pm$ SEM from at least 3 independent experiments. * $P < 0.05$.

Figure 2 The RGDKGE-containing collagen fragment activates $\beta 3$ integrin and enhances the levels of nuclear YAP. **A:** Quantification of SKOV-3 cell binding to collagen peptide P2 in the presence of non-specific control antibody (NT), function blocking anti- $\beta 3$ -integrin antibody (Anti- $\beta 3$) or function blocking anti- $\beta 1$ -integrin antibody (Anti- $\beta 1$). **B:** Quantification of ID8-VEGF cell binding to collagen peptide P2 in the presence of non-specific control antibody (NT), function blocking anti- $\beta 3$ -integrin antibody (Anti- $\beta 3$) or function blocking anti- $\beta 1$ -integrin antibody (Anti- $\beta 1$). **C:** Whole cell lysates were prepared from ID8-VEGF cells stimulated with control peptide CP or collagen peptide P2 for 5 minutes. Example of western blot analysis for the

levels of the phosphorylated $\beta 3$ integrin (p- $\beta 3$ Integrin, Tyr 747), total $\beta 3$ integrin (T- $\beta 3$ Integrin) or loading control Actin. **D:** Quantification of the mean fold change in phosphorylated $\beta 3$ integrin from at least 3 independent experiments. **E:** Whole cell lysates were prepared from ID8-VEGF cells stimulated with collagen peptide P2 in presence of non-specific control antibody (Ab Cont) and anti-RGDKGE collagen fragment antibody (Mab XL313). Example of western blot analysis for the levels of the phosphorylated $\beta 3$ integrin (p- $\beta 3$ Integrin, Tyr 747), total $\beta 3$ integrin (T- $\beta 3$ Integrin) or loading control Actin. **F:** Quantification of the mean fold change in phosphorylated $\beta 3$ integrin from at least 3 independent experiments. **G:** Nuclear fractions were prepared from ID8-VEGF cells stimulated with collagen peptide P2 in presence of non-specific control antibody (Ab Cont) and anti- $\beta 3$ integrin antibody (Anti- $\beta 3$). Example of western blot analysis for the levels of nuclear YAP or loading control TBP. **H:** Quantification of the mean fold change in nuclear YAP from at least 3 independent experiments. Data bars represent means $\pm$ SEM from at least 3 independent experiments. * $P < 0.05$.

Figure 3 Regulation of YAP phosphorylation and target gene c-Myc by the RGDKGE collagen fragment. **A:** Nuclear fractions were prepared from ID8-VEGF cells stimulated with collagen peptide P2 or control peptide CP. Left panel, example of western blot analysis for the levels of nuclear YAP or loading control TBP. Right panel, quantification of the mean fold change in nuclear YAP from at least 3 independent experiments. **B:** Nuclear fractions were prepared from ID8-VEGF cells stimulated with collagen peptide P2 in presence of non-specific control antibody (Ab Cont) or anti-RGDKGE collagen fragment antibody (Mab XL313). Left panel, example of western blot analysis for the levels of nuclear YAP or loading control TBP. Right panel, quantification of the mean fold change in nuclear YAP from at least 3 independent

experiments. **C:** ID8-VEGF cells were transfected with either a non-targeting control shRNA (Cont-NT) or YAP specific shRNA (YAP/KD). Quantification of the mean relative fold change in YAP mRNA from 4 independent experiments. **D:** Quantification of the mean fold change in c-Myc mRNA levels in ID8-VEGF cells stimulated with control peptide CP or collagen peptide P2 from at least 3 independent experiments. **E:** Quantification of the mean fold change in c-Myc mRNA levels in ID8-VEGF cells stimulated with collagen peptide P2 in the presence of non-specific control antibody (Ab Cont) or anti-RGDKGE collagen fragment antibody (Mab XL313) from at least 3 independent experiments. **F:** Cytosolic fractions were prepared from ID8-VEGF cells stimulated with control peptide CP or collagen peptide P2. Example of western blot analysis for the levels of phosphorylated YAP (p-YAP Ser-127), total YAP (T-YAP) or loading control Actin. **G:** Quantification of the mean fold change in phosphorylated YAP from at least 3 independent experiments. **H:** Cytosolic fractions were prepared from ID8-VEGF cells stimulated with collagen peptide P2 in the presence of non-specific control antibody (Ab Cont) or anti-RGDKGE collagen fragment antibody (Mab XL313). Example of western blot analysis for the levels of phosphorylated YAP (p-YAP Ser-127), total YAP (T-YAP) or loading control Actin. **I:** Quantification of the mean fold change in phosphorylated YAP (p-YAP Ser-127) from at least 3 independent experiments. Data bars represent means $\pm$ SEM from at least 3 independent experiments. * $P < 0.05$.

Figure 4 Regulation of LATS1 phosphorylation by the RGDKGE collagen fragment. **A:** Cytosolic fractions were prepared from ID8-VEGF cells stimulated with collagen peptide P2 in the presence of non-specific control antibody (Ab Cont) or anti-RGDKGE collagen fragment antibody (Mab XL313). Example of western blot analysis for the levels of phosphorylated

LATS1 (P-LATS1-Ser 1079) or loading control Actin. **B:** Quantification of the mean fold change in phosphorylated LATS1 (P-LATS1-Ser 1079) from at least 3 independent experiments. **C:** Nuclear fractions were prepared from ID8-VEGF cells stimulated with collagen peptide P2 in the presence of control (DMSO) or Src inhibitor (Src In). Example of western blot analysis for the levels of nuclear YAP or loading control TBP. **D:** Quantification of the mean fold change in nuclear YAP from at least 3 independent experiments. **E:** Nuclear fractions were prepared from ID8-VEGF cells stimulated with collagen peptide P2 in the presence of the Src inhibitor (Src In) and in the presence of non-specific control antibody (Ab Cont) or anti-RGDKGE collagen fragment antibody (Mab XL313). Example of western blot analysis for the levels of nuclear YAP or loading control TBP. **F:** Quantification of the mean fold change in nuclear YAP from at least 3 independent experiments. **G:** Cytosolic fractions were prepared from ID8-VEGF cells stimulated with collagen peptide P2 in the presence of the Src inhibitor (Src In) and the presence of non-specific control antibody (Ab Cont) or anti-RGDKGE collagen fragment antibody (Mab XL313). Example of western blot analysis for the levels of phosphorylated LATS1 (p-LATS1-1079) or loading control Actin. **H:** Quantification of the mean fold change in phosphorylated LATS1 (p-LATS1-1079) from at least 3 independent experiments. Data bars represent means $\pm$ SEM from at least 3 independent experiments. * $P < 0.05$.

Figure 5 Inhibition of the RGDKGE collagen fragment inhibits ovarian tumor growth. **A:** Quantification of the dose dependent effects of anti-RGDKGE collagen fragment antibody (Mab XL313) or non-specific control antibody (Ab Cont) on the growth of SKOV-3 tumors in vivo. Arrow indicates start of treatment with antibodies. Data points indicate mean $\pm$ SEM tumor volumes (N=8 per group). **B:** Quantification of the effects of anti-RGDKGE collagen fragment

antibody (Mab XL313) or non-specific control antibody (Ab Cont) on SKOV-3 tumor angiogenesis. Data bars indicate mean $\pm$ SEM number of CD31 positive vessels per 200X field within 5 independent fields from 5 different tumors per condition. **C:** Quantification of the effects of anti-RGDKGE collagen fragment antibody (Mab XL313) or non-specific control antibody (Ab Cont) on the subcutaneous growth of ID8-VEGF tumors in vivo. Data bars indicate mean $\pm$ SEM tumor volume (N=8 per group) measured on day 42. **D:** Representative example of the differential abdominal distension of ID8-VEGF cell injected mice treated for 13-weeks with anti-RGDKGE collagen fragment antibody (Mab XL313) or non-specific control antibody (Ab Cont). **E:** Quantification of the effects of anti-RGDKGE collagen fragment antibody (Mab XL313) or non-specific control antibody (Ab Cont) on the relative volume of ascites. Data bars indicate mean $\pm$ SEM ascites fluid volume (N=5 per group) measured at 13-weeks. **F:** Quantification of the effects of anti-RGDKGE collagen fragment antibody (Mab XL313) or non-specific control antibody (Ab Cont) on the wet weights of mouse diaphragms. Data bars indicate mean $\pm$ SEM wet weight (N=5 per group) measured at 13-weeks. * P<0.05.

Figure 6 Mab XL313 mediated inhibition of ovarian tumor growth depends on YAP. **A:** Quantification of the effects of anti-RGDKGE collagen fragment antibody (Mab XL313) or non-specific control antibody (Ab Cont) on the growth of control transfected SKOV-3 tumor cells (Non-targeting SKOV-3) in vivo. Arrow indicates start of treatment with antibodies. Data points indicate mean $\pm$ SEM tumor volumes (N=8 per group). **B:** Quantification of the effects of anti-RGDKGE collagen fragment antibody (Mab XL313) or non-specific control antibody (Ab Cont) on the growth of SKOV-3 tumor cells in which YAP levels have been knocked down (YAP/KD-SKOV-3). Arrow indicates start of treatment with antibodies. Data points indicate mean $\pm$ SEM

tumor volumes (N=8 per group). * $P < 0.05$. **C:** Working model of a potential mechanism by which the endogenously generated RGDKGE collagen fragment may regulate the nuclear accumulation of YAP.

Figure 1.

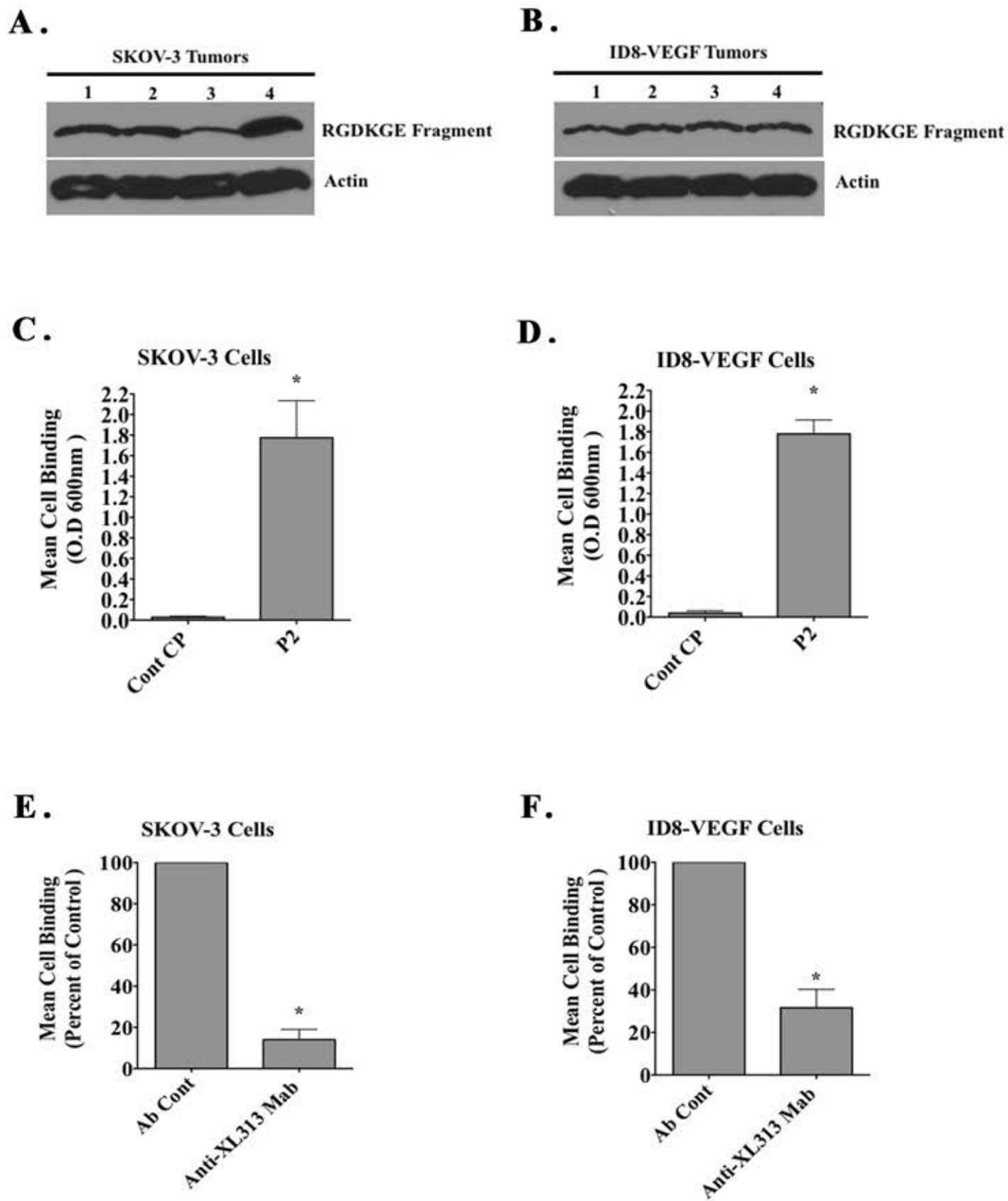


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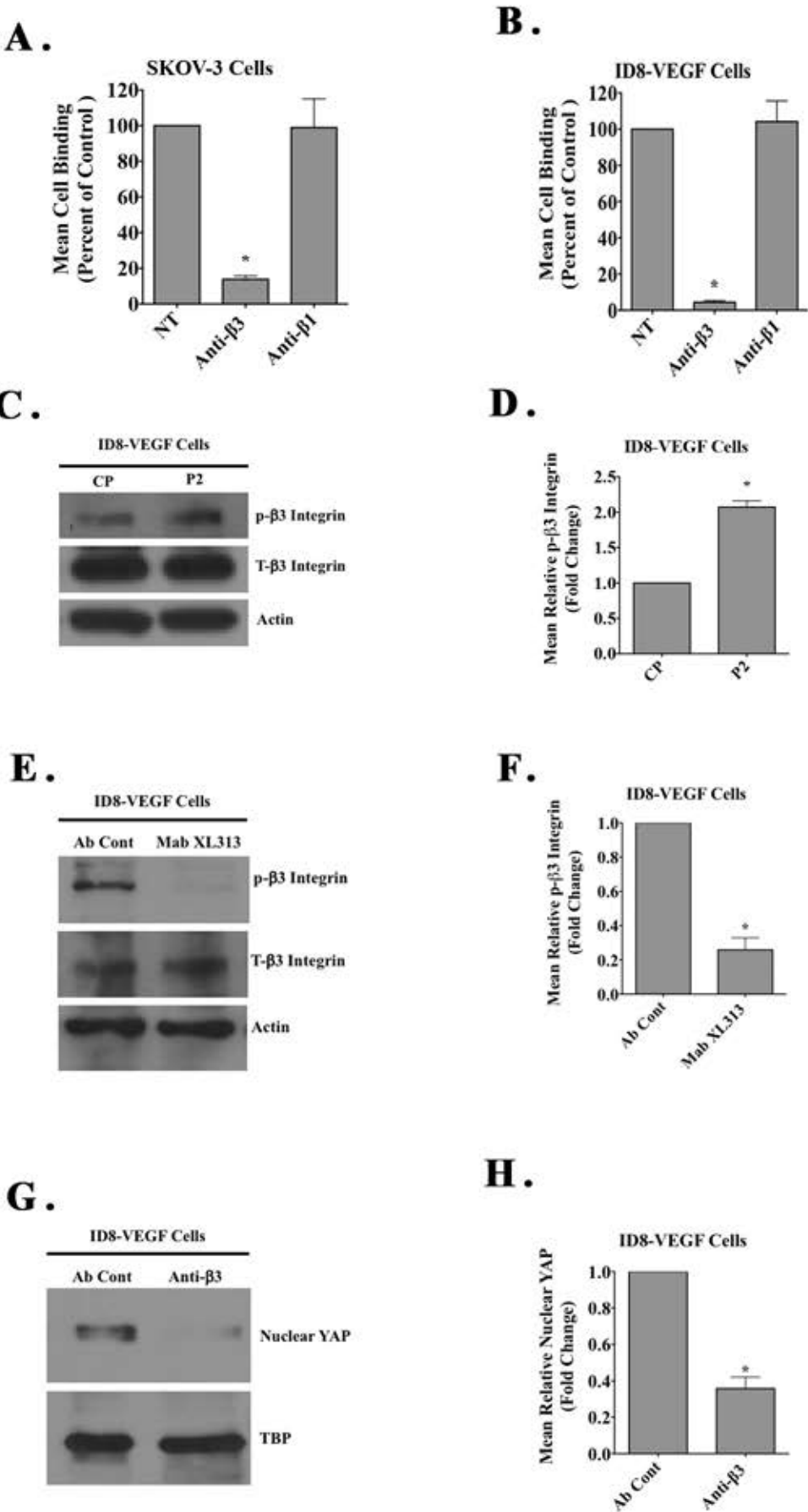


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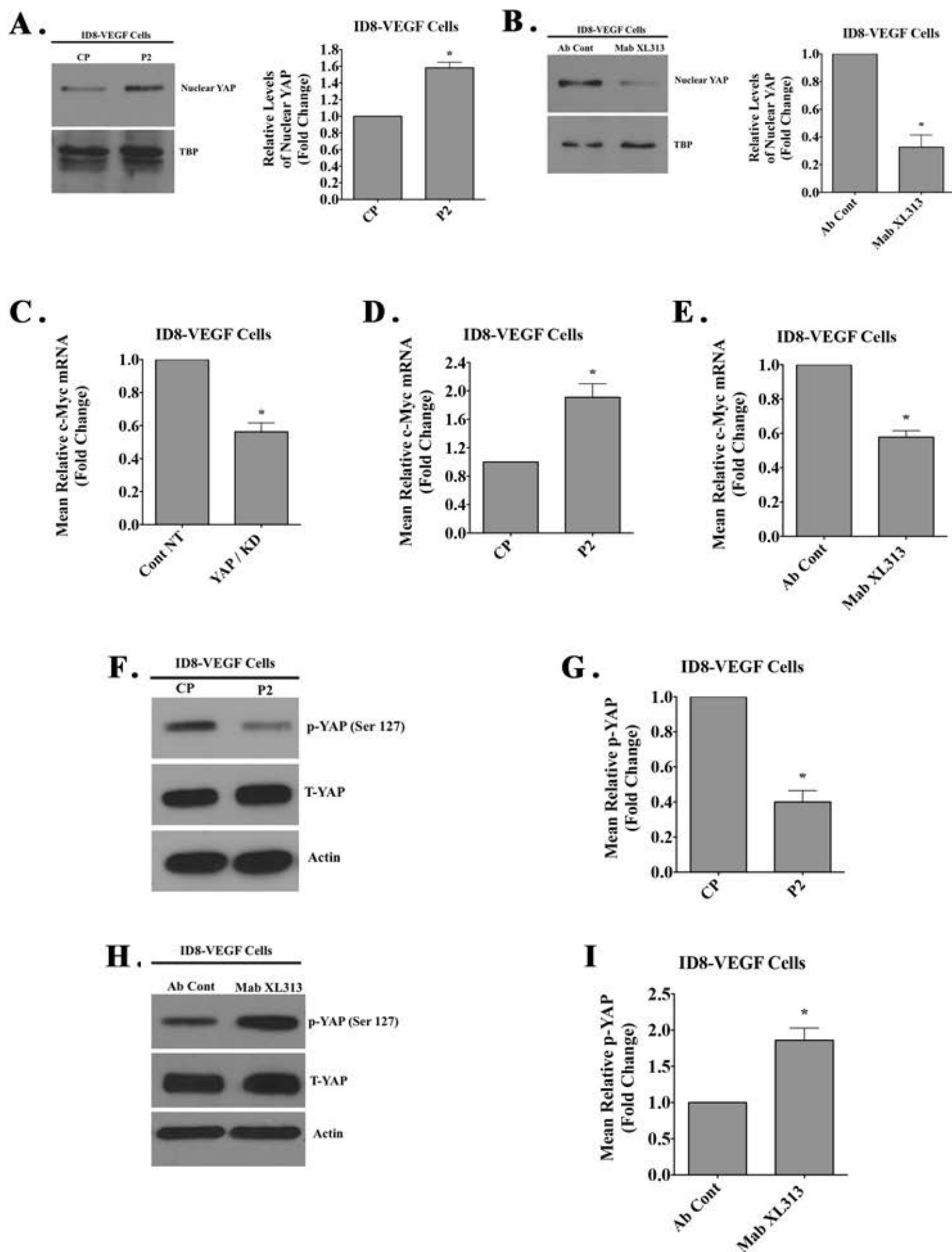


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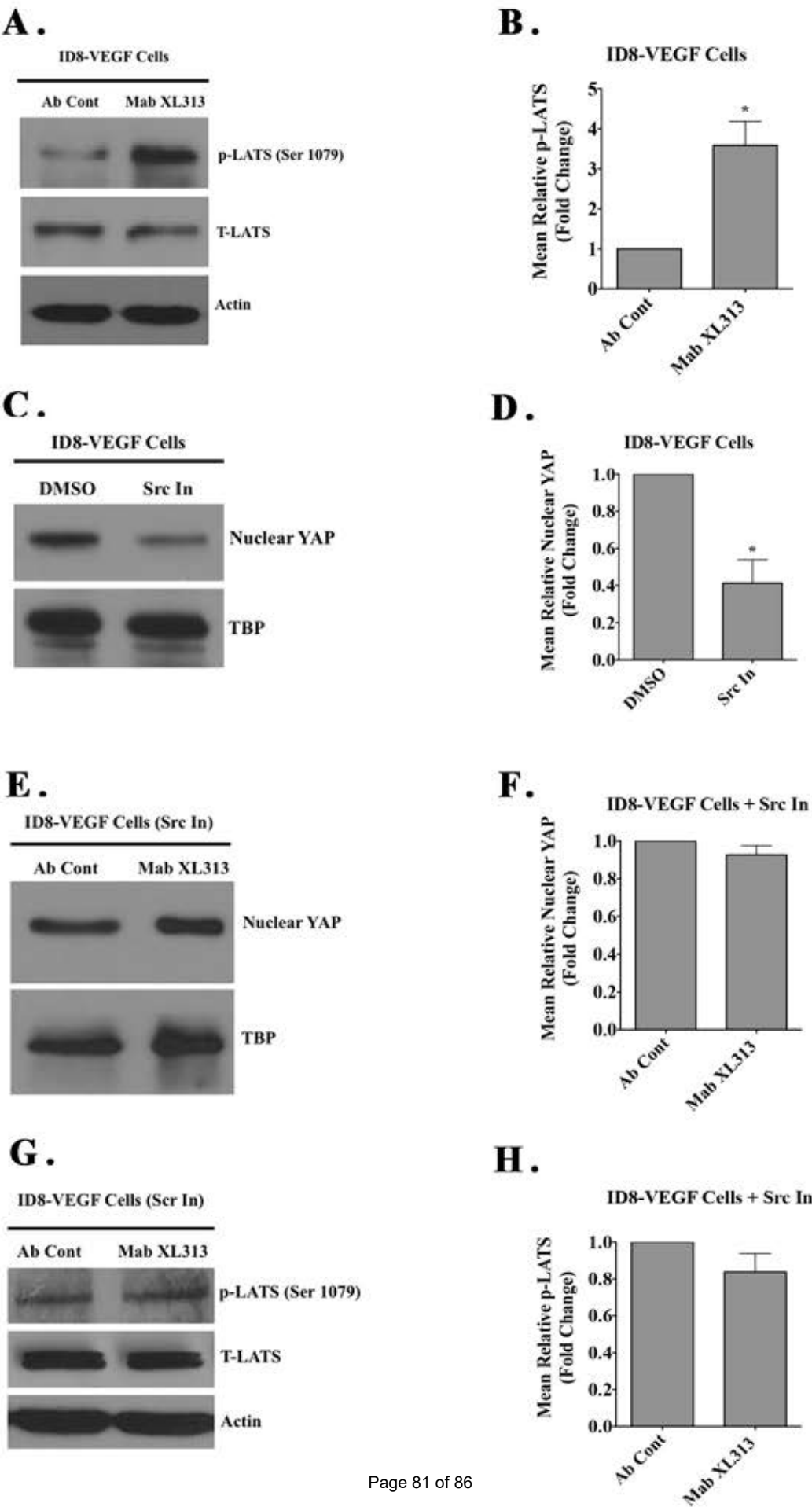


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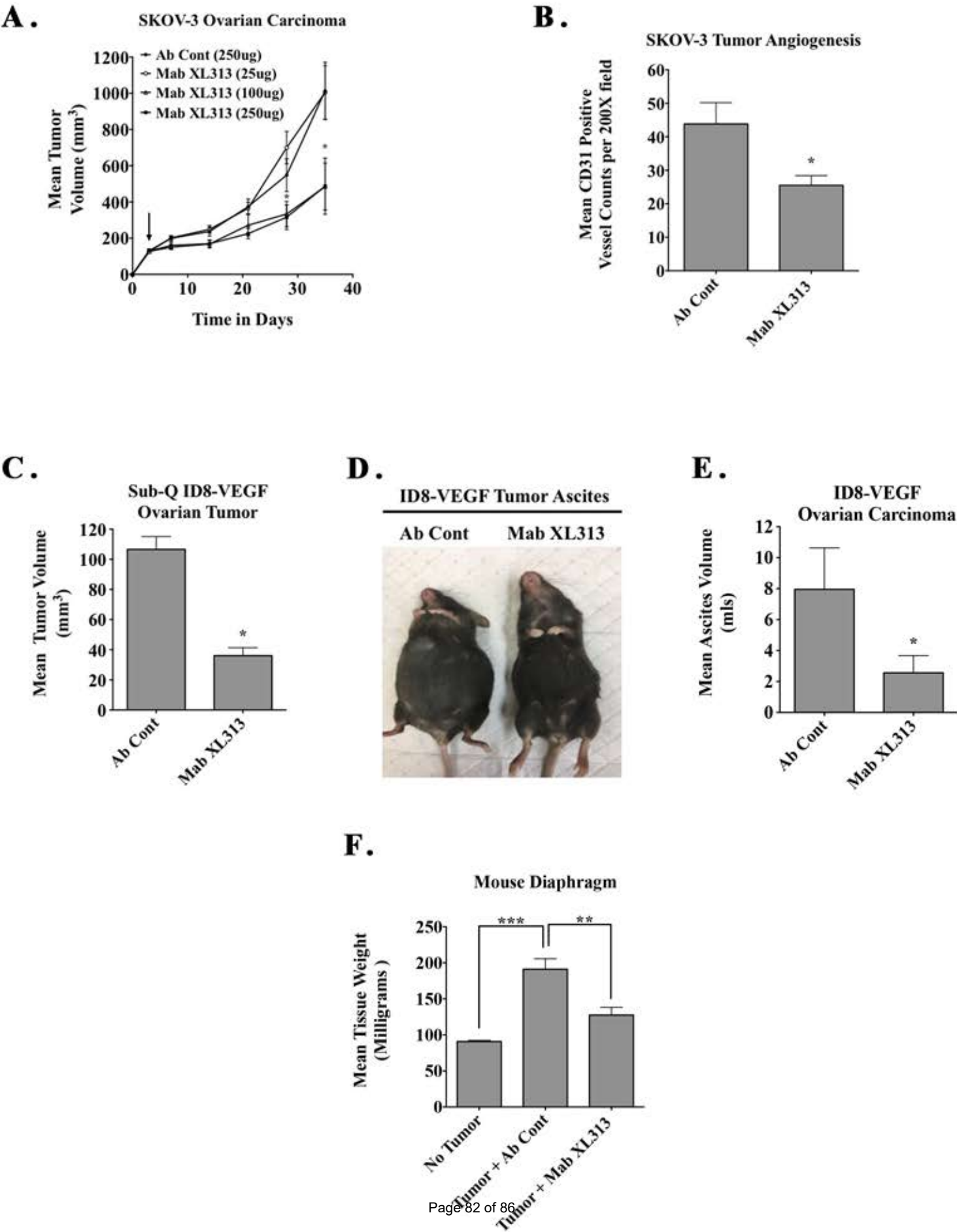
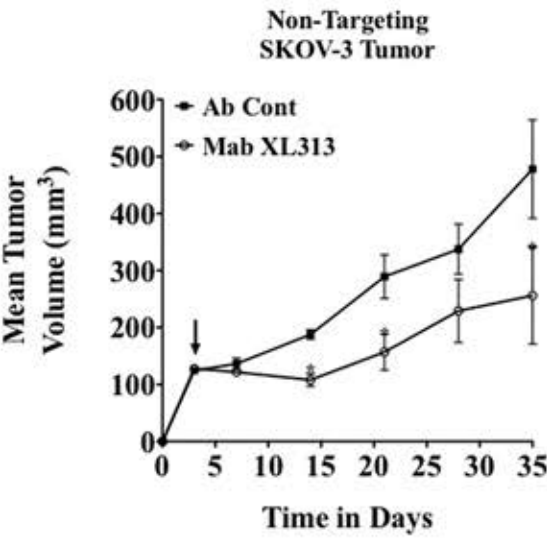
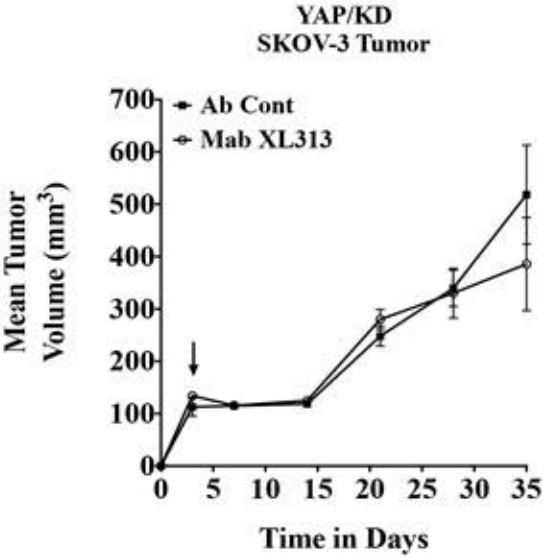


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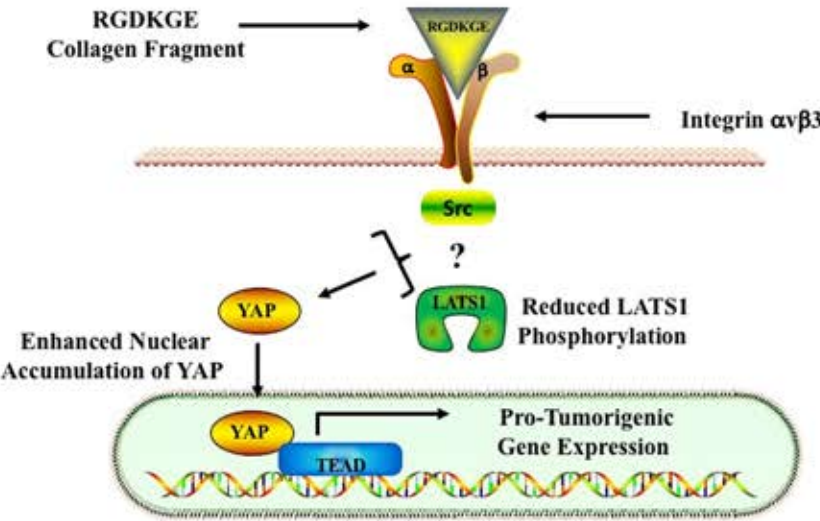
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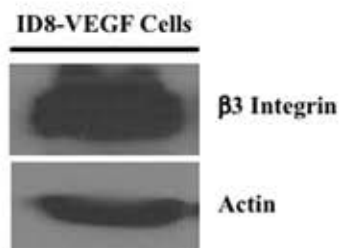


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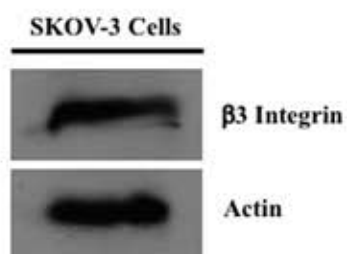


Supplemental Figure 1

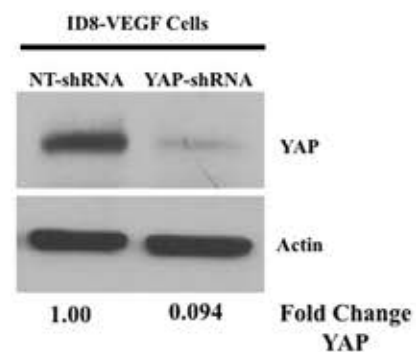
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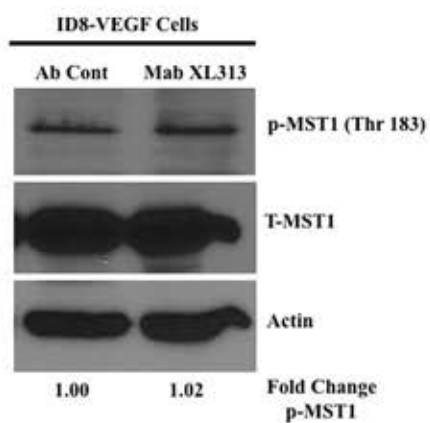
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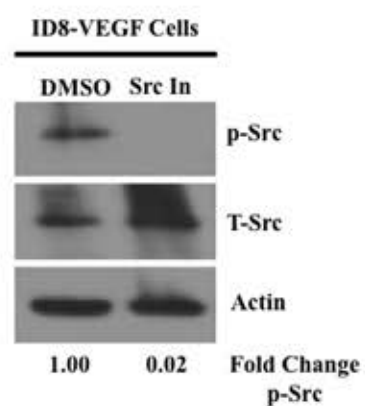
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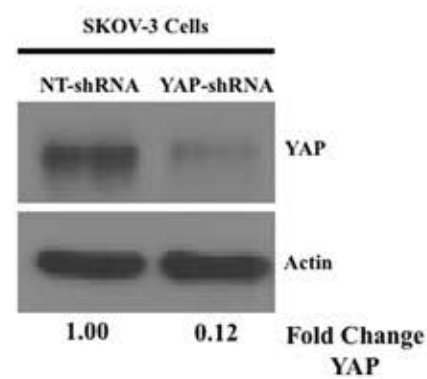
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Supplemental Figure Legend.

Figure S1A and B. Detection of $\beta 3$ integrin in ovarian tumor cell lines. Whole cell lysates were prepared from sub-confluent murine ID8-VEGF cells (A) and human SKOV-3 cells using RIPA buffer and analyzed for the expression of integrin $\beta 3$. Representative examples of western blot for integrin $\beta 3$ or loading control Actin.

Figure S1C. Quantification of relative levels of YAP in Yap knock down ID8-VEGF cells. Whole cell lysates were prepared from sub-confluent control shRNA transfected (NT-shRNA) and *Yap*- shRNA transfected ID8-VEGF ovarian tumor cells using RIPA buffer and analyzed for YAP expression. Representative example of western blot for YAP or loading control Actin. Relative fold change in levels of protein expression were determined by Image J.

Figure S1D. Effects of Mab XL313 antibody on the relative levels of phosphorylated MST1 in ID8-VEGF cells. Briefly, ID8-VEGF cells were serum starved in suspension for 30 minutes then incubated in the presence Mab XL313 or non-specific control antibody for 30 minutes and seeded on RGDKGE-containing collagen peptide P2 and cytoplasmic fractions were prepared for analysis. Representative example of western blot for phosphorylated MST1 (Thr 183), total MST1 or loading control Actin. Relative fold change in levels of protein expression were determined by Image J.

Figure S1E. Effects of the Dasatinib on the relative levels of phosphorylated Src in ID8-VEGF cells. Briefly, ID8-VEGF cells were treated (1uM) with the Src inhibitor Dasatinib (Src

In) or control (DMSO). Whole cell lysates prepared using RIPA buffer. Representative example of western blot for phosphorylated Src (Tyr 416), total Src or loading control Actin. Relative fold change in levels of protein expression were determined by Image J.

Figure S1F. Quantification of relative levels of YAP in Yap knock down SKOV-3 cells.

Whole cell lysates were prepared from sub-confluent control shRNA transfected (NT-shRNA) and YAP- shRNA transfected SKOV-3 ovarian tumor cells using RIPA buffer and analyzed for YAP expression. Representative example of western blot for YAP or loading control Actin. Relative fold change in levels of protein expression were determined by Image J.