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Award Number: W81XWH-09-1-0083

TITLE: Chemotherapeutic Targeting of Fibulin-5 to Suppress Breast Cancer Invasion and Metastasis Simulated by Transforming Growth Factor-Beta

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REPORT DATE: January 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-01-2012	Annual	15 Dec 2010 - 14 Dec 2011

4. TITLE AND SUBTITLE
Chemotherapeutic Targeting of Fibulin-5 to Suppress Breast Cancer Invasion and MetastasisSimulated by Transforming Growth Factor-Beta

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0083

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Case Western Reserve University
Cleveland, OH 44106

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The primary objective of this application is to establish how Fibulin-5 enhances the oncogenic activities of TGF-beta, particularly its ability to stimulate breast cancer invasion and metastasis. We hypothesized that inactivating Fibulin-5 function will prevent the conversion of TGF-beta from a suppressor to a promoter of breast cancer growth and invasion, thereby alleviating breast cancer development and progression stimulated by TGF-beta. Major findings of the past funding cycle include the ability of Fibulin-5 to (i) bind integrins on mammary epithelial cells (MECs) independent of its integrin-binding RGD motif; (ii) interact physically with TGF-beta and enhance its presentation to TGF-beta receptors; (iii) promote epithelial-mesenchymal transition in an integrin-independent manner in part by stimulating the expression of Cox-2, PAI-1, and MMP-9; (iv) enhance MEC proliferation by activating FAK and ERK1/2; and (v) induce MEC resistance to apoptosis and anoikis by stimulating NF-kappaB activation, by inducing survivin and xIAP expression, and by repressing TNF-alpha expression. Finally, we determined that Fibulin-5 expression is greatly augmented during breast cancer progression, particularly at the point when malignant MECs acquire metastatic phenotypes. This important finding implicates Fibulin-5 as a potential marker for breast cancer metastasis and reinforces the need to target Fibulin-5 chemotherapeutically in patients with metastatic disease.

15. SUBJECT TERMS
Transforming Growth Factor-beta; Fibulin-5; Breast Cancer; Metastasis; Invasion; Chemotherapy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	38	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:

Breast cancer is the second leading cause of cancer death in women in the United States. Invasion and metastasis are the most lethal characteristics of breast cancer and the leading cause of breast cancer-related death. TGF-β normally inhibits breast cancer development by preventing mammary epithelial cell (MEC) proliferation, or by inducing MEC apoptosis. Mammary tumorigenesis counteracts the tumor suppressing activities of TGF-β, thus enabling TGF-β to stimulate breast cancer invasion and metastasis. Fundamental gaps exist in our knowledge of how malignant MECs overcome the cytostatic actions of TGF-β, and of how TGF-β stimulates the development and progression of mammary tumors. These knowledge gaps have prevented science and medicine from implementing treatments effective in antagonizing the oncogenic activities of TGF-β in developing and progressing breast cancers. We recently discovered that the expression and activity of the TGF-β gene target, Fibulin-5 (FBLN5), potentiates TGF-β stimulation of invasion and epithelial-mesenchymal transition (EMT) in normal and malignant MECs *in vitro*, and more importantly, enhances the growth and pulmonary metastasis of mammary tumors in mice. Interestingly, we find that FBLN5 incorporates into active TGF-β receptor complexes in a β3 integrin-dependent manner, an event associated with the activation of intracellular signaling by TGF-β. Based on these and other compelling findings, we hypothesized that inactivating FBLN5 function will prevent the conversion of TGF-β from a suppressor to a promoter of breast cancer growth and invasion, thereby alleviating breast cancer development and progression stimulated by TGF-β. The goals of this project are to determine the molecular mechanisms that mediate incorporation of FBLN5 into active TGF-β receptor complexes, and to determine the role of FBLN5 in mediating β3 integrin and Src activation, leading to oncogenic signaling by TGF-β in normal and malignant MECs. Finally, we will determine whether interdicting FBLN5 function abrogates the oncogenic activities of TGF-β and prevents its stimulation of breast cancer progression *in vivo.* These studies are important because they will *(i)* provide valuable information on how breast cancers develop and progress, and on how TGF-β promotes these processes; *(ii)* identify the signaling mechanisms and systems that mediate the oncogenic nature of TGF-β; and *(iii)* identify FBLN5 antagonists capable of alleviating the oncogenic activities of TGF-β, as well as establish their effectiveness in preventing breast cancer progression stimulated by TGF-β. Moreover, application of our findings will enable science and medicine to one day improve the prognosis and treatment of patients with metastatic breast cancer

BODY:

Overview and General Findings: The specific aims of the proposed research have not been modified. Indeed, our recently published manuscript in the journal *Carcinogenesis* [1] clearly established the importance of FBLN5 in promoting epithelial-mesenchymal transition (EMT) in normal and malignant mammary epithelial cells (MECS). Equally important, we showed that FBLN5 expression greatly enhanced the ability of TGF-β to stimulate EMT, as well as promoted its oncogenic activities in normal and malignant MECs both *in vitro* and *in vivo*. Clearly, elucidating the molecular mechanisms that enable FBLN5 to enhance oncogenic TGF-β signaling has tremendous potential to neutralize the metastasis promoting activities of this multifunctional cytokine, and as such, to ultimately improve the clinical course of breast cancer patients with metastatic disease.

Data in the scientific literature has recently established the essential role of TGF-β in regulating the activities of breast cancer-associated fibroblasts and stromal components [2-5]. Indeed, mounting evidence indicates that TGF-β promotes breast cancer progression in part *via* its reprogramming of MEC microenvironments and their cellular architectures. Moreover, TGF-β also induces desmoplastic and fibrotic reactions that elicit the formation of tense, rigid tumor microenvironments that *(i)* enhance the selection and expansion of developing mammary neoplasms, particularly that of late-stage metastatic cells, and *(ii)* predict for poor clinical outcomes in breast cancer patients. Our previous published studies established FBLN5 as an important stromal-produced secreted factor that regulates tumor development in mice [6-9]. Thus, we characterized changes in the fibroblast transcriptome elicited by FBLN5, or by FBLN5 plus TGF-β. Microarray analyses identified 1181 genes whose expression is regulated by FBLN5, and an additional 1675 genes whose expression is regulated by

TGF-β. Differential expression of 14 individual genes was verified by semi-quantitative real-time PCR. Downregulated FBLN5 gene targets included *a)* BB503935; *b)* pleckstrin-homology domain-containing family A member; *c)* transglutaminase-2; and *d)* Rho GTPase activating protein 24. Upregulated FBLN5 gene targets included *a)* BB533736; *b)* BB831146; *c)* HoxD9; *d)* thrombospondin-1; *e)* collagen type XI; *f)* angiopoietin-1; *g)* cysteine-rich protein 61; *h)* Dkk3; *i)* fibromodulin; and *j)* HoxD10. Oncomine analyses showed the expression of fibromodulin to be upregulated in human breast cancers, and as such, we further characterized the activities of this novel FBLN5 gene target. In doing so, we found that fibromodulin expression greatly enhanced the coupling of TGF-β to Smad2/3 and AP-1 activation, while simultaneously abrogating both basal and TGF-β-stimulated NF-κB activation in fibroblasts. Importantly, we observed fibromodulin expression to stabilize that of the NF-κB inhibitory protein, IκBα. We further determined that fibromodulin stabilized IκBα expression by activating JNK and CK-II, which inactivate calpain and its proteolytic activity against IκBα. Thus, in addition to inhibiting NF-κB activity in fibroblasts, the activation of this fibromodulin-dependent pathway promotes apoptosis in fibroblasts. Even more strikingly, Oncomine analyses showed the expression of fibromodulin to be reduced at metastatic sites relative to nonmetastatic lesions in gastric cancers, head and neck cancers, and sarcomas. These analyses also found aberrantly low fibromodulin expression to associate with reduced overall survival rates in patients with cancers of the brain, breast, lung, and blood. Our findings related to this novel FBLN5:fibromodulin signaling axis were published in February 2001 by the *Journal of Biological Chemistry* (vol 286, pages 6414-6422). **Clinically, chemotherapeutic targeting of this pathway may offer novel inroads into alleviating the oncogenic activities of TGF-β in breast cancer stroma.**

Over the course of the past funding cycle, we have potentially discovered some spectacular insights into a longstanding paradox that has confounded *TGF-β Biologists* over the last decade – *i.e.,* how do gains [1, 10-21] and losses [5, 22, 23] of TGF-β function both drive breast cancer metastasis within the same late-stage breast cancers? As will be discussed in greater detail below, we recently observed hypoxia to be sufficient in inactivating the TGF-β signaling, presumably as a means to circumvent the ability of TGF-β to induce apoptosis in hypoxic MECs. Along these lines, we also observed hypoxia to significantly upregulate FBLN5 expression, which mediates survival signaling and prevents MEC apoptosis elicited by hypoxia. These findings are potentially paradigm changing and provide novel and innovative insights into the inherent plasticity employed by late-stage breast cancer cells to facilitate their development and metastatic progression. **These important findings are now being prepared to publication.**

Based on our findings presented below, we remain convinced that our analyses of noncanonical and oncogenic effectors targeted by FBLN5 and TGF-β will enable the development of safer, more directed chemotherapies capable of phenotypically normalizing and reverting the malignant behaviors of developing and progressing breast cancers.

***PLEASE NOTE*: Figures and findings previously presented as part of our BC084651 mid-term and annual reports are indicated in "blue text – e.g., Fig. 1." Newly generated figures and findings over the course of the last funding cycle are indicated in "red text – e.g., Fig. 2."**

Task-Specific Findings:

Task 1: ***Determine the molecular mechanisms that mediate incorporation of FBLN5 into active TGF-β receptor complexes.*** We previously engineered normal NMuMG and metastatic 4T1 cells to stably express β3 integrin or its inactive mutant, D119A-β3 integrin [10-12]. Our previously published studies demonstrated the function of β3 integrin in promoting oncogenic TGF-β signaling, including its ability to stimulate EMT and pulmonary metastasis of breast cancer cells [10-12]. We recently introduced wild-type FBLN5 and its RGE-mutant, which we demonstrated previously to prevent FBLN5 from ligating integrins on endothelial cells [7]. The functional characteristics of these FBLN5 and β3 integrin manipulations on MEC behavior in response to TGF-β are quite interesting and will be discussed below (*see Task 2).* With respect to the primary objective of Task 1 – *i.e.,* to identify the molecular determinants that mediate incorporation of FBLN5 into active TGF-β receptor complexes,

and more importantly, to determine the impact of disrupting the formation of these complexes on normal and malignant MEC response to TGF-β – our preliminary data indicate that FBLN5 is capable of binding β3 integrin on MECs independent of its integrin-binding RGD motif. Indeed, we find that MEFs derived from FBLN5-deficient embryos respond poorly to TGF-β, and that re-expression of either wild-type FBLN5 or RGE-FBLN5 molecules in these FBLN5-deficient MEFs significantly enhance MEF response to TGF-β. Thus, our findings to date suggest that FBLN5 may incorporate into TGF-β receptor complexes independent of traditional integrin-binding activities. Alternatively, FBLN5 may incorporate into TGF-β receptor complexes in a manner wholly independent of β3 integrin. With respect to the former possibility, we now are optimizing the expression and purification systems necessary to isolate various recombinant FBLN5 mutants, including full-length wild-type and RGE-mutant FBLN5 molecules, as well as those mutants that lack the N-terminal Pro-rich domain (*i.e.,* ΔPro), the entire N-terminal domain (*i.e.,* ΔNT), and the entire globular C-terminal domain (*i.e.,* ΔCT) or those that only contain the N-terminal (*i.e.,* NT-FBLN5) or C-terminal (*i.e.,* CT-FBLN5). FBLN5 mutants found to incorporate into TGF-β receptor complexes will then be subjected to gross- and fine-deletion analyses, followed by Ala-scanning mutagenesis to elucidate the molecular determinants that mediate FBLN5 association with TGF-β receptors. We fully expect to possess engineered FBLN5 molecules that are incapable of supporting oncogenic TGF-β signaling by the completion of Year 2, and to complete a thorough characterization of their impact on TGF-β signaling and breast cancer cell behavior during Year 3.

Because our initial studies of FBLN5 incorporation into TGF-β receptor complexes showed that wild-type and RGE-FBLN5 were both capable of capturing β3 integrin in immunocomplex assays, we began to consider the possibility that FBLN5 may incorporate into TGF-β receptors in an integrin-independent fashion. In support of this notion, we found that FBLN5 bears striking homology to members of LTBP (latent TGF-β-binding proteins) family of proteins, particularly in their calcium-binding EGF-like repeats. Thus, we hypothesized that FBLN5 may bind directly to TGF-β, which then pulls FBLN5 into TGF-β receptor complexes. Accordingly and quite surprisingly, we used three separate and distinct binding protocols to show unambiguously that FBLN5 does indeed interact physically with active TGF-β independent of whether FBLN5 can bind to integrins (*i.e.,* wild-type FBLN5 and RGE-FBLN5 bind indistinguishably to active TGF-β). **This finding represents a major advance for TGF-β and FBLN5 biologists, and may in fact explain why FBLN5-deficient MEFs are unresponsive to TGF-β**. Indeed, our findings indicate that FBLN5 may function in binding directly to TGF-β and facilitating its presentation and/or incorporation to inactive TGF-β receptor complexes, resulting in enhanced transmembrane signaling initiated by TGF-β. Accordingly, MECs engineered to overexpress FBLN5 exhibit significantly elevated levels of Smad2/3 activity as compared to their GFP-expressing counterparts, a finding consistent with FBLN5 functioning to present and enhance autocrine TGF-β signaling in normal and malignant MECs. We have now engineered MECs to produce various FBLN5 mutants to map the domains operant in mediate its interaction with TGF-β. After affirming which regions of FBLN5 bind TGF-β1, we will immediately generate FBLN5 mutants that lack this domain/motif to assess how preventing FBLN5 from binding TGF-β impacts normal and malignant MEC response to TGF-β both *in vitro* and *in vivo.* As above, we fully expect to complete this exciting and important task during Year 2, and to complete a thorough characterization of their impact on TGF-β signaling and breast cancer cell behavior during Year 3

Task 2: ***Determine the role of FBLN5 in mediating β3 integrin and Src activation, leading to oncogenic signaling by TGF-β in normal and malignant MECs.*** The primary objective of Task 2 is to identify FBLN5 effectors operant in mediating oncogenic signaling by TGF-β. In this regard, we have found that wild-type and RGE-FBLN5 are both capable of promoting partial EMT phenotype in normal MECs (**Fig. 1A**). Moreover, we find FBLN5 expression to be significantly upregulated in 4T1 progression series, which is an established mouse model of triple-negative breast cancer (TNBC; **Fig. 1B**). Interestingly, we also observed the combination of FBLN5 and β3 integrin to significantly enhance the proliferative *(data not shown)* and invasive (**Fig. 1C**) potential of normal MECs, a response that was not recapitulated in MECs co-expressing RGE-FBLN5 and β3 integrin. In addition,

the combined expression of FBLN5 and β3 integrin greatly attenuated the sensitivity of MECs to the cytostatic activities of TGF-β. The enhanced response of MECs to FBLN5 also correlated with its ability to significantly augment the activation of FAK and ERK1/2 in these same cells *(data not shown)*. Thus, FBLN5 expression induced by TGF-β in normal and malignant MECs appears to play a significant role in mediating its growth promoting activities in MECs. We recently developed the only *in vitro* assay that wholly recapitulates the phenomena underlying the "TGF-β Paradox" during mammary tumorigenesis [16, 24], which converts the actions of TGF-β from that of a tumor suppressor to a tumor promoter (*see* [25-28]). We exploited this unique 3D-organotypic culture system to further explore the function of FBLN5 in promoting an invasive phenotype in normal MECs. In doing so, we propagated parental NMuMG cells or their derivatives engineered to stably express either FBLN5, β3 integrin, or both transgenes in combination in the absence or presence of TGF-β under compliant or rigid 3D-organotypic culture conditions. **Figure 2** shows that relative to parental (*i.e.,* GFP/YFP) NMuMG cells, those engineered to express β3 integrin formed substantially larger (by 275%) and densely packed acinar structures, while those expressing FBLN5 formed substantially smaller (by 65%) organoids. Interestingly, NMuMG cells that expressed both transgenes formed acinar structures whose size was also substantially larger (by 195%) than their parental counterparts and in many respects resembled those of β3 integrin-expressing NMuMG cells (**Fig. 2**). Thus, β3 integrin stimulates acinar growth, while FBLN5 inhibits this event in a manner that can be neutralized by expression of β3 integrin. Despite the dramatic differences in their relative growth rates, all four NMuMG derivatives readily responded to the cytostatic activities of TGF-β and formed diminutive organoids when propagated in continued presence of this cytokine (**Fig. 2**). When cultured under rigid conditions, all NMuMG derivatives acquired a branched morphology that was potentiated by β3 integrin expression (**Fig. 2**). Quite surprisingly, FBLN-expressing NMuMG cells formed unique linear and irregularly-branched structures indicative of a highly invasive phenotype (**Fig. 2**). Moreover and in contrast to their parental and β3 integrin-expressing expressing counterparts, FBLN5-expressing NMuMG cells were uniquely resistant to the apoptotic inducing activities of TGF-β under rigid microenvironmental conditions (**Fig. 2**). Moreover, the survival promoting activities of FBLN5 are wholly coupled to the ability of FBLN5 to activate Src *(data not shown*). Taken together, these new findings offer some potentially important insights into the role of FBLN5 in MECs, suggesting that FBLN5 may function as an inhibitor of acinar growth and development in compliant microenvironments. However, these findings also suggest that FBLN5 may sense and respond to mechanically rigid and tense microenvironmental conditions, resulting in the acquisition of invasive and survival phenotypes.

Along these lines and in stark contrast to its effects in fibroblasts [9], we find that FBLN5 greatly enhances basal and TGF-β-stimulated NF-κB activity in normal (**Fig. 3A**) and malignant *(data not shown)* MECs in part *via* promoting increased degradation of IκBα (**Fig. 3B**). Moreover, these FBLN5-dependent activities require signaling inputs initiated by Src *(data not shown)*. Indeed, we recently found that EMT induced by TGF-β initiates a pro-survival gene expression profile *(data not shown)*, such that MECs that survive the EMT process are more resistant to apoptosis and anoikis. Given our published work that FBLN5 promotes EMT in normal and malignant MECs [1], we reasoned that FBLN5 expression would also promote survival signaling in these same cells. Accordingly, we now find that FBLN5 greatly suppresses TNF-α expression (by 90%) in normal MECs *(data not shown)*, while simultaneously stimulating that of the *(i)* survival factors, survivin and xIAP *(data not shown)*; *(ii)* angiogenic and EMT molecule, Cox-2 *(data not shown)*; *(iii)* pro-metastatic molecule, PAI-1 *(data not shown)*; and *(iv)* pro-invasion and EMT-molecule, MMPs 2, 3, and 9 *(see below)*. In addition, we further observed FBLN5 expression to be sufficient in inhibiting Caspase-3/7 activation by TNF-α in normal MECs (**Fig. 4**). Along theses lines, neoadjuvants and conventional chemotherapies can accelerate disease progression and metastasis *via* a hypoxia-induced EMT [29-33]. We too have demonstrated the ability of the c-Abl antagonist, Gleevec (Imatinib) to elicit EMT programs and disease progression of TNBCs [24]. Given these apparent associations between hypoxia and metastatic progression, we addressed the question as to whether hypoxia augmented or attenuated TGF-β signaling in normal and malignant MECs. **Figure 5A** shows that 4T1 cells readily responded to TGF-β when propagated in traditional 2D-cultures under normoxic

conditions, but not when there were cultured under hypoxic conditions. These cells are subject to extensive autocrine TGF-β signaling [13-15, 20, 21], which enhances breast cancer development and progression. As shown in **Fig. 5B**, the ability of hypoxia to inhibit gene transcription appeared to be specific for that mediated by TGF-β, as CMV-driven gene expression was unaffected by changes in oxygen tension. Similar effects of normoxia and hypoxia on TGF-β signaling were also observed metastatic breast cancer cells propagated in 3D-organotypic cultures (**Fig. 5C & D**). Interestingly, we also found FBLN5 expression to be significantly induced by hypoxia (**Fig. 6**). A primary goal of our research is to develop novel means to eradicate TNBC. A major limitation to eradicating TNBCs reflects the inability to create a xenograft model that recapitulates TNBC development and metastatic progression. We have recently devised the means to overcome this barrier by transforming normal MECs (*i.e.,* NMuMG cells) *via* their enforced expression of EGFR, which is upregulated in TNBCs and predicts for poor patient prognosis [34, 35]. We refer to these transformed MECs as "NME cells," which readily form nonmetastatic tumors in mice [20]. Importantly, we can induce NME tumors to acquire metastatic phenotypes by first stimulating them to undergo EMT *in vitro* prior to their implantation into the mammary fat pads of mice [20]. In fact, mice injected with pre-EMT NME cells are cured following primary tumor resection, while animals injected with post-EMT NME cells rapidly develop lethal disease recurrence upon removal of their primary tumors. Additionally, following their stimulation with TGF-β, metastatic NME cells acquire a TNBC phenotype by losing expression of ER-α and PR. Importantly, the phenotypes of individual pre- and post-EMT NME cells are stable following *ex vivo* isolation, thereby providing a unique, powerful, and innovative *in vitro* and *in vivo* system to *"deconstruct"* the molecular and spatiotemporal underpinnings that govern TNBC development, metastatic progression, and disease recurrence. **Figure 7A** shows that parental and FBLN5-expressing NME cells exhibit similar growth characteristics and sensitivity to cytostasis mediated by TGF-β. However, culturing these same cells under hypoxic conditions demonstrated the ability of FBLN5 to significantly promote the growth of NME cells as compared to that of their parental counterparts (**Fig. 7B**). Likewise, enforced expression of FBLN5 conferred NMuMG cells resistance to hypoxia-driven apoptosis (**Fig. 8**), presumably by preventing Caspase-3 cleavage and activation (**Fig. 9**), and by stimulating NF-κB (**Fig. 10**). **Collectively, these findings identify FBLN5 as a novel promoter of breast cancer survival in hypoxic tumor conditions, and as such, bolster the notion that chemotherapeutic targeting of FBLN5 may provide a novel means to eliminate metastatic breast cancers prior to their initial exit and dissemination from their primary tumors. We will test this hypothesis, as well as determine the molecule mechanism that elicits transient inactivation of TGF-β signaling in hypoxic mammary tumors.**

In addition, we have begun manipulating the expression of these FBLN5 gene targets in normal and malignant MECs to access their role in regulating MEC response to TGF-β both *in vitro* and *in vivo.* Initial targets are members of the MMP family of proteases. Indeed, we observed FBLN5 to strongly induce the expression of MMPs 2, 3, and 9 in normal (**Fig. 11**) and metastatic (**Fig. 12**). Interestingly, whereas the ability of FBLN5 to activate NF-κB transpires through an integrin-independent pathway (**Fig. 3**), the coupling of FBLN5 to MMP expression clearly requires signaling inputs from integrins. For instance, **Figure 13** shows that FBLN5, but not its mutant RGE counterpart (*i.e.,* cannot ligate integrins), readily and potently induces the expression of MMPs 2, 3, and 9. Surprisingly, expression of β3 integrin inhibited the ability of FBLN5 to promote MMP expression, a reaction that was not recapitulated by expression of the nonfunctional β3 integrin mutant, D119A-β3 integrin. Thus, while FBLN5 clearly binds β3 integrin, this event serves to neutralize MMP expression stimulated by FBLN5. Mechanistically, the ability of FBLN5 to induce MMP expression in normal MECs transpires through the activation of MAP kinases and Src, as well as require Ca^{++}-dependent signaling inputs (**Fig. 14**). Our findings in **Figure 15** wholly support this idea and also show that the integrin effectors, FAK and Pyk2, are necessary for FBLN5 stimulation of MMP expression in NMuMG cells. In addition to binding to αvβ3 integrin, FBLN5 also ligates αvβ5 and α9β1 integrins, suggesting that the coupling of FBLN5 to MMP expression way proceed through other integrin heterodimers operant in binding FBLN5. Along these lines, we have recently determined that FBLN5 induces MMP expression through a β1 integrin- and ERK1/2-dependent pathway (**Fig. 16**). Indeed, neutralizing antibodies against β3 integrin were ineffective in altering the coupling of FBLN5 MMP

expression, which contrasts sharply to the dramatic reduction in MMP expression observed following administration of either neutralizing β1 integrin antibodies (**Fig. 16**), or following cellular depletion of β1 integrin expression (**Fig. 17**). Likewise, we also observed FBLN5 to induce MMP expression to be dependent upon the PTK activity of EGFR, as determined by the ability of the EGFR inhibitor, AG1478 to abrogate MMP expression stimulated by FBLN5 (**Fig. 18A**). Similar antagonism of IGF-1R signaling by administration of AG1024 had no effect on MMP expression induced by FBLN5 (**Fig. 18B**). Mechanistically, these FBLN5-specific events transpire through its stabilization of EGFR expression in MECs (**Fig. 18C**), as well as through its ability to enhance the coupling of EGFR to ERK1/2 in these same MECs (**Fig. 19 & 20**). The latter response is also entirely dependent upon signaling inputs arising from β1 integrin (**Fig. 20**), suggesting that FBLN5 coordinates that the oncogenic crosstalk that exists between β1 integrin and EGFR in mammary tumors.

Finally, two recent studies have linked the induction of EMT by TGF-β to its regulation of protein translation, a reaction comprised of a TGF-β:AKT2:hnRNP E1 signaling axis that enables the production of Dab2 and ILEI [36, 37]. Mechanistically, AKT2-mediated phosphorylation of hnRNP E1 dissociates this molecule from transcripts containing the BAT elements, thereby initiating the synthesis of proteins operant in driving EMT. Additionally, rendering NMuMG cells deficient in hnRNP E1 is sufficient to induce an EMT program [36, 37] in a manner reminiscent of that observed by the overexpression of FBLN5 in these same cells [1]. Moreover, MECs that have emerged from EMT programs universally express robust levels of FBLN5 independent of EMT-initiating agent [38], suggesting an essential role for FBLN5 in driving EMT programs. Along these lines, we recently obtained hnRNP E1-deficient NMuMG cells from Dr. Philip H. Howe (MUSC) and find that these MECs house aberrantly elevated (by ~2000-fold) levels of FBLN5 transcript (**Fig. 21A**) and protein *(data not shown)*. Consistent with the ability of FBLN5 to induce MMP-9 expression (**Fig. 11-18**), we observed hnRNP E1-deficient NMuMG cells to express dramatically upregulated levels of MMP-9 (**Fig. 21B**). Finally, **Fig. 22** shows that FBLN5 induces Dab2 expression in part *via* a β1 integrin-dependent manner, thereby implicating FBLN5 as an important player responsible in mediating the actions of hnRNP E1 in transitioning MECs. We are now testing this important hypothesis by depleting FBLN5 expression in hnRNP E1-deficient cells to gauge the extent to which this ECM protein drives EMT and survival signaling, as well as induces MMP expression during the metastatic progression of malignant MECs.

These findings are a major advance to the fibulin field, and we now are rapidly extending these findings to the aforementioned normal and malignant MECs engineered to express all combinations of wild-type and mutant FBLN5 and β3 integrin molecules.

Task 3: ***Determine whether interdicting FBLN5 function abrogates the oncogenic activities of TGF-β and prevents its stimulation of breast cancer progression in vivo***. The primary objective of Task 3 is to establish the effectiveness of abolishing FBLN5 function and its subsequent incorporation into active TGF-β receptor complexes to prevent breast cancer progression and metastasis induced by TGF-β. As mentioned above, this past year saw us identify a variety of novel FBLN5 gene targets, as well as uncover two potentially important tumor promoting functions for FBLN5, namely its ability to facilitate the presentation of TGF-β to its receptors and its potential to induce survival signaling in normal and malignant MECs. These findings are consistent with a role of FBLN5 in driving EMT (**Fig. 1**; [1]), invasion (**Fig. 1**; [1]), and metastasis in late-stage breast cancers [1]. Accordingly, FBLN5 expression is increased robustly in the murine 4T1 progression series from weakly tumorigenic 67NR cells to fully metastatic 4T1 cells (**Fig. 1**). Along these lines, we recently determined that FBLN5 promotes EMT in MECs *via* a β1 integrin-dependent manner (**Fig. 23**), thereby implicating a novel interplay that governs the balance between β1 and β3 integrins in mediating breast cancer development and metastatic progression stimulated by TGF-β. To further explore this idea, we performed initial pilot studies to determine whether overexpression of FBLN5 in NMuMG cells would be sufficient to induce their formation of mammary tumors in nude mice. Unfortunately, tumor development was not induced by FBLN5 expression, indicating that aberrant expression of the ECM molecule is not sufficient to transform MECs and drive tumor development. However, FBLN5 expression greatly enhanced the development and progression of NME tumors produced in mice (**Fig. 24**). In the next year, we will rapidly test these FBLN5 functions using malignant, nonmetastatic 67NR and malignant,

highly metastatic 4T1 cells that will be engineered to stably express FBLN5 mutants that fail to bind and present TGF-β to its receptors, as well as those construct derivatives of these breast cancer cell lines whose expression of FBLN5 target genes has been positively and negatively manipulated. Afterward, the impact of these manipulations on primary tumor growth and metastasis will be assessed in syngeneic Balb/C mice.

KEY RESEARCH ACCOMPLISHMENTS:

- Mammary tumorigenesis upregulates FBLN5 expression, particularly at the point when breast cancer cell acquire metastatic phenotypes
- A novel FBLN5 gene signature has now been identified and established
- The FBLN5 gene target, fibromodulin, suppresses NF-κB activity by stabilizing IκBα expression
- Stabilization of IκBα transpires via JNK and CK-II activation, which conspire to inactivate calpain and its proteolytic activity against IκBα
- Activation of this fibromodulin signaling axis promotes apoptosis
- FBLN5 interacts with β3 integrin in an RGD-independent fashion
- FBLN5 binds TGF-β, leading to its enhanced presentation to TGF-β receptors and elevated autocrine TGF-β signaling in normal and malignant MECs
- FBLN5 and β3 integrin promote normal and malignant MEC proliferation, a cellular response coupled to FAK and ERK1/2 activation by FBLN5
- RGE-FBLN5 and β3 integrin fail to induce MEC proliferation
- FBLN5 induces survival signaling in normal and malignant MECs in part by strongly activating NF-κB
- Survival signaling by FBLN5 is also coupled to its ability to suppress TNF-α expression, and to induce that of survivin and xIAP
- FBLN5 potentially induces breast cancer cell EMT, migration, and invasion by upregulating the expression of Cox-2, PAI-1, and MMPs 2, 3 and 9
- β1 integrin is essential for coupling FBLN5 to ERK1/2 activation, which subsequently induces MMP expression in normal and malignant MECs.
- β1 integrin is also essential in mediating EMT induced by FBLN5.
- FBLN5 promotes normal and malignant MEC survival under hypoxic conditions.
- Hypoxia selectively inactivates the TGF-β signaling system, presumably circumventing the tumor suppressing activities of this cytokine.
- FBLN5 induces MMP expression *via* an EGFR- and ERK1/2-dependent mechanism.
- FBLN5 stabilizes EGFR expression, leading to elevated ERK1/2 signaling and enhanced tumor development in mice.

REPORTABLE OUTCOMES:

Schiemann Laboratory Publications Acknowledging Support of BC084651:

Keshamouni, V.G. and **Schiemann, W.P.** (2009) EMT in Tumor Metastasis: A Method to the Madness. **Future Oncology** *5,* 1109-1111.

Wendt, M.K., Allington, T.M. and **Schiemann, W.P.** (2009) Mechanisms of epithelial-mesenchymal transition by TGF-β in normal and malignant cells. **Future Oncology** *5,* 1145-1168.

Tian, M. and **Schiemann, W.P.** (2009) The TGF-β paradox in human cancer: An update. **Future Oncology** *5,* 259-271.

Wendt, M.K., Smith, J.A. and **Schiemann, W.P.** (2009) p130Cas is required for mammary tumor growth and TGF-β-mediated metastasis through regulation of Smad2/3 activity. **Journal of Biological Chemistry** *284,* 34145-34156 *See Faculty of 1000 (http://f1000biology.com/article/id/1168476)

Taylor, M.A., Parvani, J.G. and **Schiemann, W.P.** (2010) The pathophysiology of EMT stimulated by TGF-β. **J Mammary Gland Biol Neoplasia** *15:* 169-190. *20 consecutive months as a "Top 5" journal download.

Wendt, M.K., Smith, J.A. and **Schiemann, W.P.** (2010) TGF-β-induced epithelial-mesenchymal transition facilitates oncogenic epidermal growth factor receptor signaling in breast cancer. **Oncogene** *29:* 6485-6498. *Featured in "Mammary Cell News" volume 2.33, September 2, 2010.

Tian, M., Neil, J.R. and **Schiemann, W.P.** (2010) TGF-β and the hallmarks of cancer. **Cell Signal** *23:* 951-962.

Allington, T.M. and **Schiemann, W.P.** (2011) The Cain and Abl of epithelial-mesenchymal transition and TGF-β in mammary epithelial cells. **Cells Tissues Organs** *193:* 98-113.

Lee, Y-H. and **Schiemann, W.P.** (2010) Fibromodulin suppresses nuclear factor-κB activity by inducing the delayed degradation of IκBα *via* a JNK-dependent pathway coupled to fibroblast apoptosis. **J Biol Chem** *286:* 6414-6422.

Taylor, M.A., Amin, J., Kirschmann, D.A. and **Schiemann, W.P.** (2011) Lysyl oxidase contributes to mechanotransduction-mediated regulation of transforming growth factor-β signaling in breast cancer cells. **Neoplasia** *13:* 406-418. *Cover and featured article (http://www.neoplasia.com/toc.php?vol=13&no=5)

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Wendt, M.K., Molter, J., Flask, C.A. and **Schiemann, W.P.** (2011) *In vivo* dual substrate bioluminescent imaging. **J Vis Exp** *56:* e3245, DOI: 10.3791/3245.

Wendt, M.K., Taylor, M.A., Schiemann, B.J. and **Schiemann, W.P.** (2011) Downregulation of epithelial cadherin is required to initiate the metastatic outgrowth of breast cancer. **Mol Biol Cell** *22:* 2423-2435. *See Faculty of 1000 (http://f1000.com/11598960#evaluations)

Wendt, M.K., Tian, M. and **Schiemann, W.P.** (2011) Deconstructing the mechanisms and consequences of TGF-β-induced EMT during cancer progression. **Cell Tissue Res** *344:* in press.

Taylor, M.A., Lee, Y-H and **Schiemann, W.P.** (2012) Role of TGF-β and the tumor microenvironment during mammary tumorigenesis. **Gene Expr** *16*: in press.

Invited Seminars Presented by Dr. Schiemann Acknowledging Support of BC084651:

Schiemann, W.P. (2009) Oncogenic TGF-β signaling in breast cancer. **UC-Davis Cancer Center,** Sacramento, CA. (May 14, 2009).

Schiemann, W.P. (2009) Oncogenic TGF-β signaling in breast cancer. **Case Comprehensive Cancer Center,** Cleveland, OH. (July 16, 2009).

Schiemann, W.P. (2009) Activated Abl kinase inhibits oncogenic TGF-β signaling, EMT, and tumorigenesis in mammary tumors. **The EMT International Association's 4th International Meeting on "Epithelial-Mesenchymal Transition,"** Tucson, AZ. (September 23, 2009).

Schiemann, W.P. (2009) The Abl and Cain of TGF-β signaling. **Department of Pharmacology,** Case Western Reserve University, Cleveland, OH. (October 5, 2009).

Schiemann, W.P. (2010) The Cain and Abl of EMT and TGF-β signaling in mammary epithelial cells. *AACR Special Conference on "EMT and Cancer Progression and Treatment,"* Arlington, VA. (March 1, 2010).

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Schiemann, W.P. (2010) Oncogenic TGF-β signaling in breast cancer. *Eppley Cancer Institute, University of Nebraska Medical Center,* Omaha, NE (October 28, 2010).

Schiemann, W.P. (2010) The Cain and Abl of EMT and TGF-β signaling in Breast Cancer. *Translational Genomics Research Institute (TGen),* Phoenix, AZ (November 8, 2010).

Schiemann, W.P. (2010) Oncogenic TGF-β Signaling in breast cancer. *Lerner Research Institute, Cleveland Clinic Foundation,* Cleveland, OH (December 7, 2010).

Schiemann, W.P. (2011) Oncogenic TGF-β signaling in breast cancer. *Department of Biological Chemistry, Johns Hopkins University,* Baltimore, MD (January 25, 2011).

Schiemann, W.P. (2011) Oncogenic TGF-β signaling in breast cancer. *Department of Human and Molecular Genetics and the VCU Institute of Molecular Medicine, Virginia Commonwealth University,* Richmond, VA (April 12, 2011).

Schiemann, W.P. (2011) Mechanisms of EMT and metastasis stimulated by TGF-β. *FASEB Summer Research Conference on "The TGF-β Superfamily: Signaling In Development and Disease,"* Barga, Italy (August 23, 2011).

Schiemann, W.P. (2011) Mechanisms of EMT and metastasis stimulated by TGF-β. *Holden Comprehensive Cancer Center, University of Iowa,* Iowa City, IA (November 29, 2011).

Schiemann, W.P. (2012) Oncogenic TGF-β signaling in breast cancer. *Rammelkamp Research Conferences, Rammelkamp Center for Education and Research, MetroHealth,* Cleveland, OH (February 14, 2012).

Schiemann, W.P. (2012) Oncogenic TGF-β signaling during metastatic progression of triple-negative breast cancers. *Department of Chemistry, Purdue University,* West Lafayette, IN (February 20, 2012).

CONCLUSION:

Our findings have clearly established new biological and pathological paradigms for FBLN5 and TGF-β. Importantly, we continue to *(i)* elucidate the mechanisms whereby FBLN5 induces oncogenic TGF-β signaling in normal and malignant MECs, and *(ii)* identify the FBLN5 effectors that contribute to the invasive and metastatic properties of TGF-β. Equally importantly, our findings have provided the first FBLN5 gene signature that underlies its biological activities, and this dataset has already uncovered fibromodulin as a novel FBLN5 gene target that regulates fibroblast survival. Our findings that FBLN5-deficient MEFs are largely unresponsive to TGF-β is exciting and may in fact be explained by our demonstration that FBLN5 binds directly to TGF-β, leading to its presentation to TGF-β receptors and the enhanced activation of autocrine TGF-β signaling in normal and malignant MECs. Our findings have also identified several novel FBLN5 effectors whose activity contributes to oncogenic TGF-β signaling. Given our recent finding that developing and progressing mammary tumors significantly upregulate their expression of FBLN5 at the point at which these tumors become metastatic, our results clearly establish FBLN5 as a new and potentially important biomarker to detect and track metastatic disease in patients with breast cancer. Moreover, the ability of FBLN5-deficiency to significantly attenuate cellular responses to TGF-β suggest that measures capable of antagonizing FBLN5 function may alleviate the initiation of oncogenic TGF-β signaling. Indeed, successful identification and implementation of FBLN5 molecules that are unable to bind and present TGF-β to its receptors on metastatic breast cancer cells holds tremendous potential to alleviate metastatic disease in breast cancer patients. Thus, translation of our findings will provide a novel set of biomarkers comprised of FBLN5 and its effectors that will be capable of predicting whether or not malignant MECs possess metastatic phenotypes. In addition, our findings will offer new inroads to target these metastatic lesions via employment of FBLN5 mutants that will suppress oncogenic TGF-β signaling in breast cancer cells. Collectively, we envision that further developing these reagents and clinical protocols will play a significant role in developing a "personalized medicine" approach tailored to treat individuals with metastatic breast cancer.

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APPENDICES:

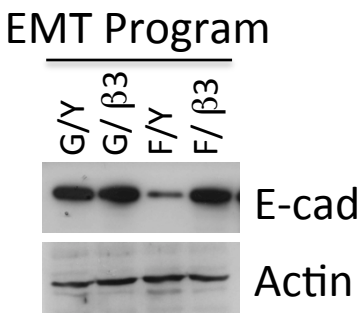
- Supporting Figures 1-24

Figure 1

FBLN5-expressing MECs Exhibit EMT and Invasive Phenotypes:

Role of FBLN5 in Breast Cancer Progression?

A



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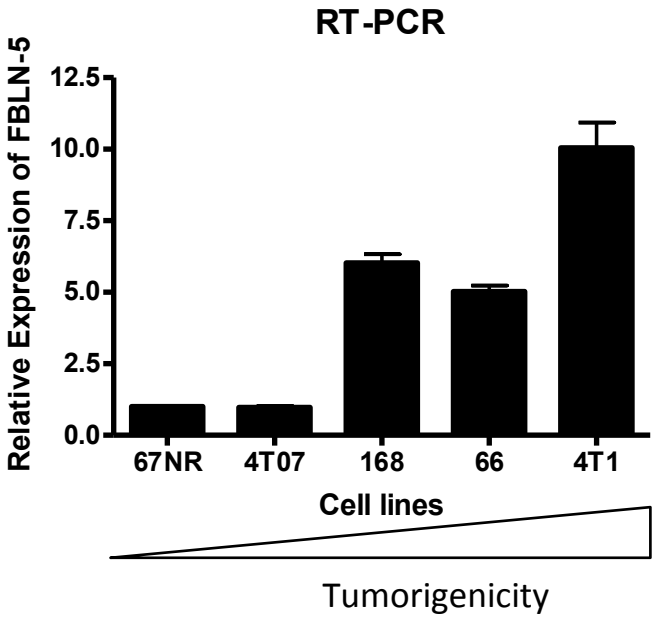

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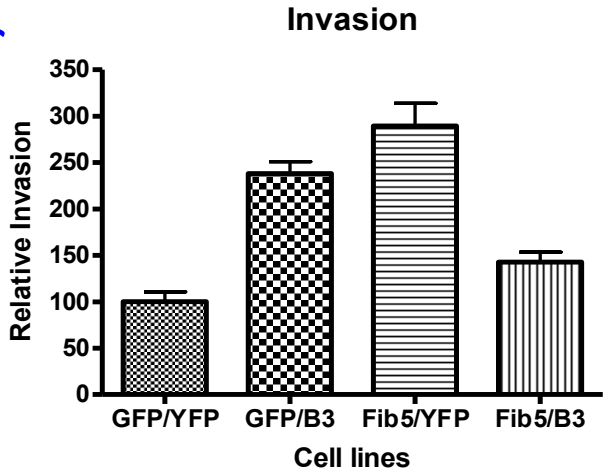
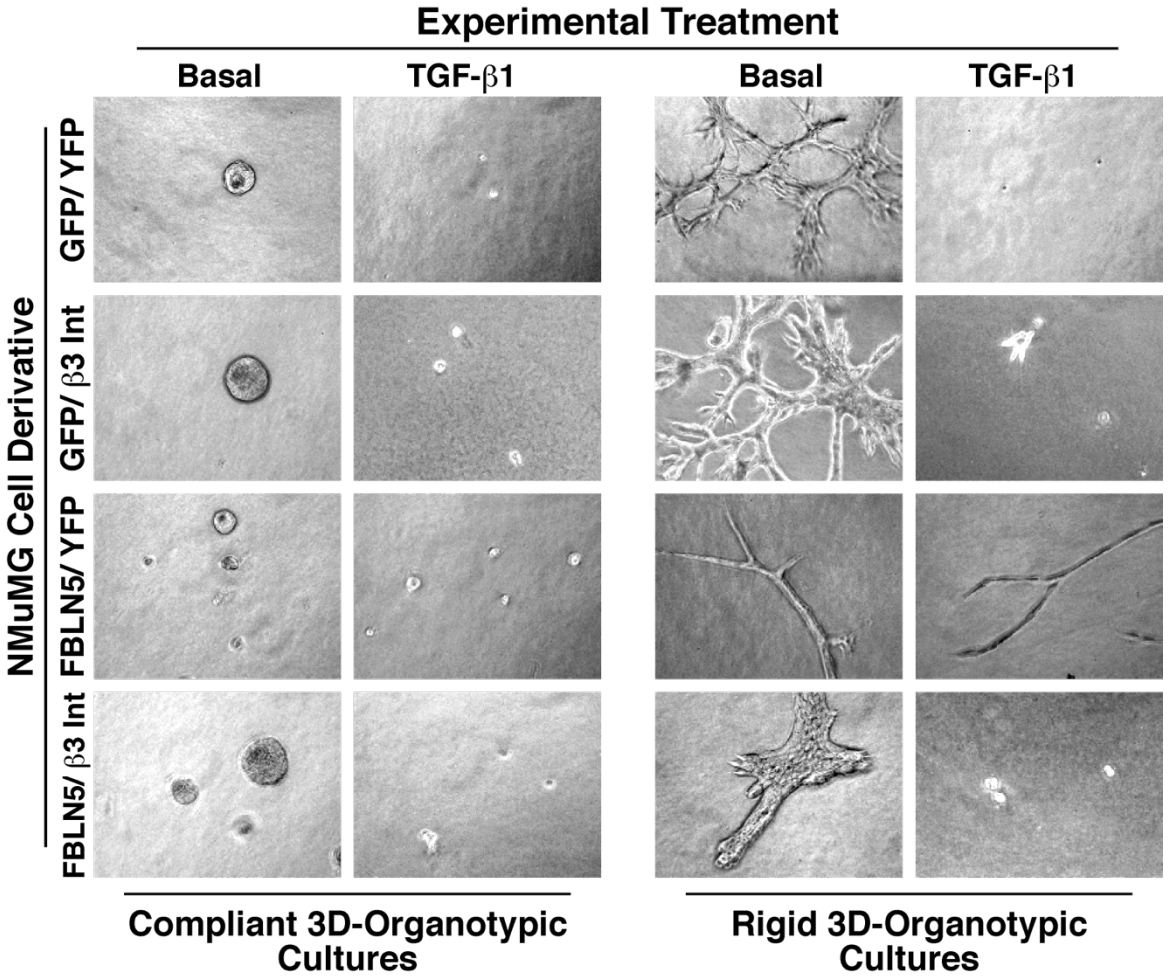


Legend

G = GFP
Y = YFP
F = FBLN5
B = β3 integrin

Figure 2

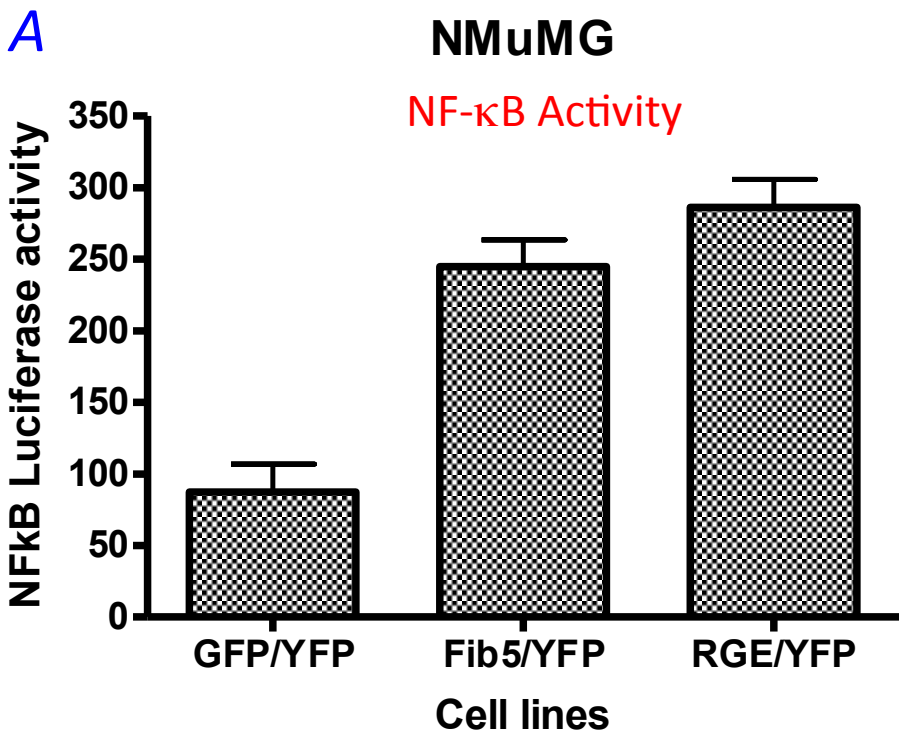
FBLN5 Induces Invasive Morphologies and Survival of MECs in Rigid Environments



NMuMG derivatives were cultured in the absence or presence of TGF-β1 (5 ng/ml) for 8 days in 3D-organotypic Cultrex matrices supplemented without (*i.e.,* compliant) or with type I collagen (3 mg/ml; *i.e.,* rigid). Bright-field images were captured and representative images from 3 independent experiments are shown.

Figure 3

FBLN5 Stimulates NF-κB Activity Independent of Integrin Binding in NMuMG Cells: *Targeting IκBα Destruction*



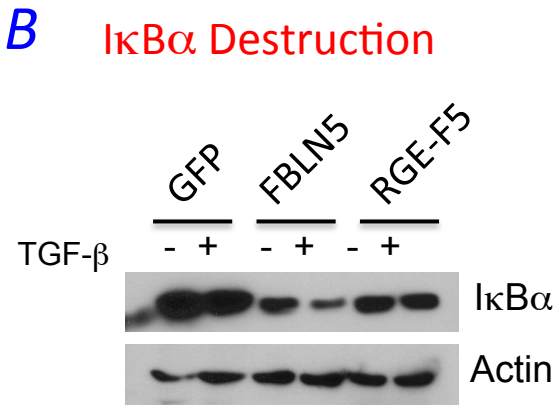


Figure 4

FBLN5 Suppresses the Coupling of TNF-α to Caspases 3 & 7 in NMuMG Cells

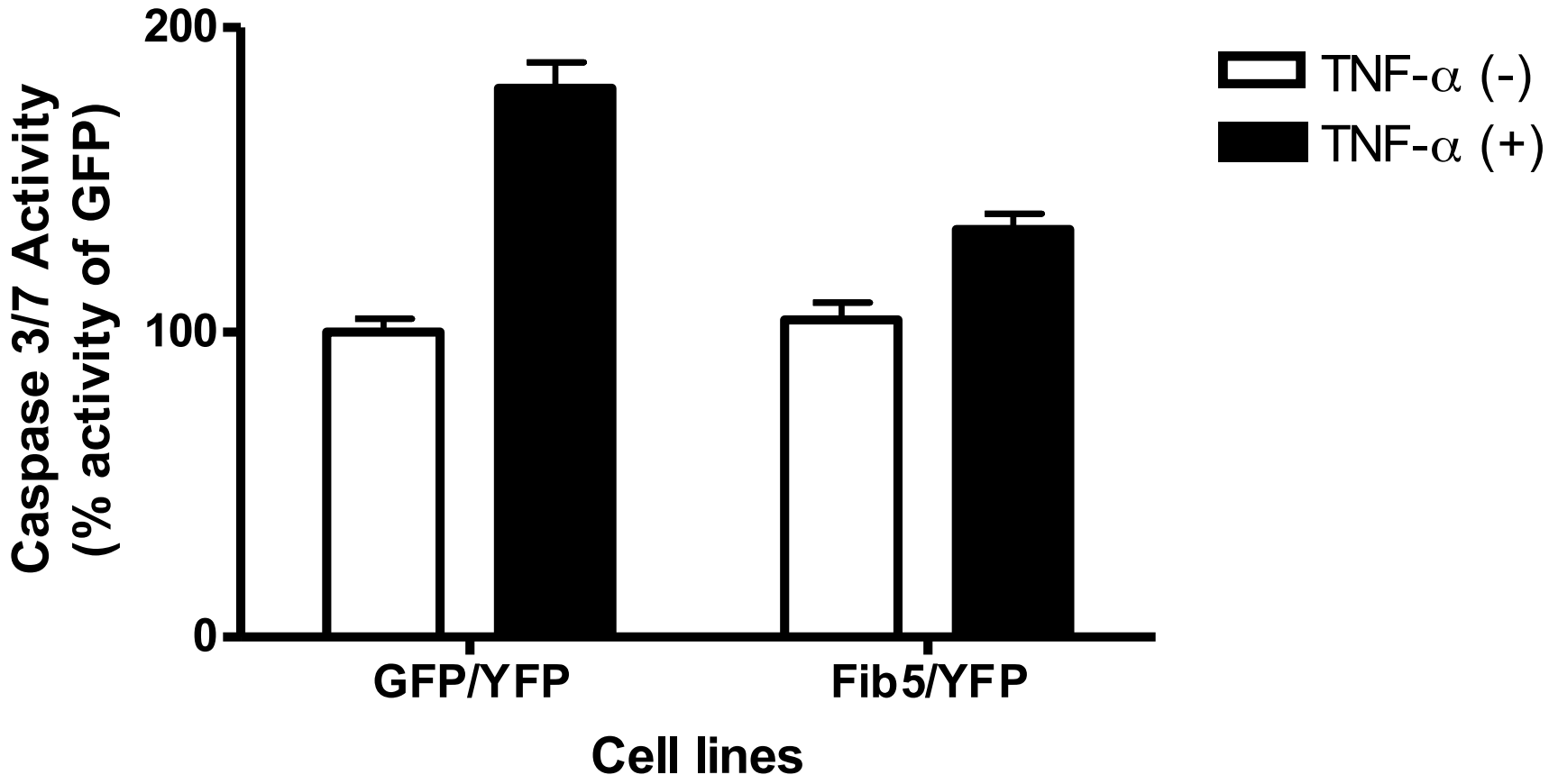


Figure 5

Hypoxia Inactivates TGF-β Signaling in Traditional 2D- and 3D-Organotypic Cultures

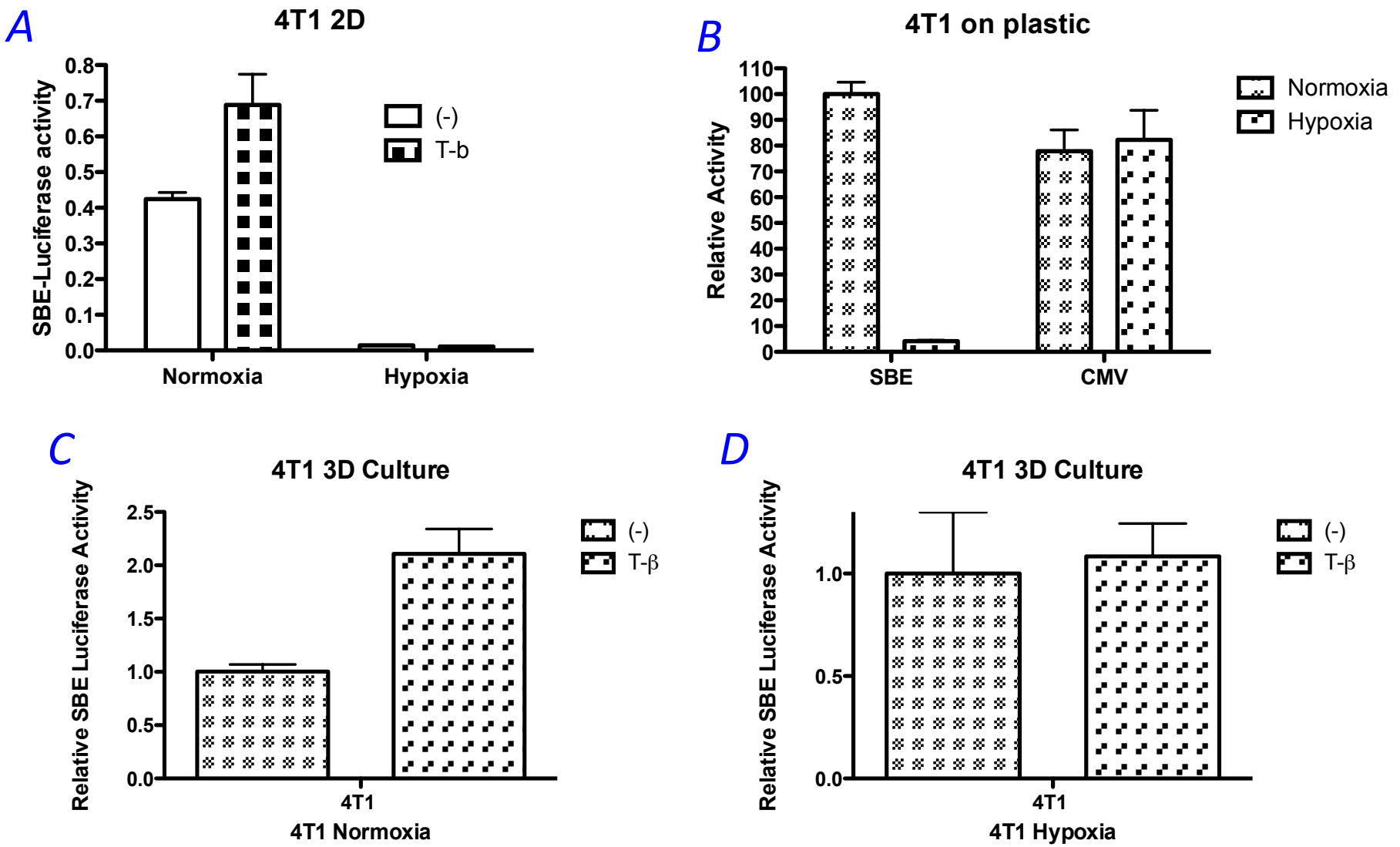


Figure 6

Hypoxia Induces FBLN5 Expression

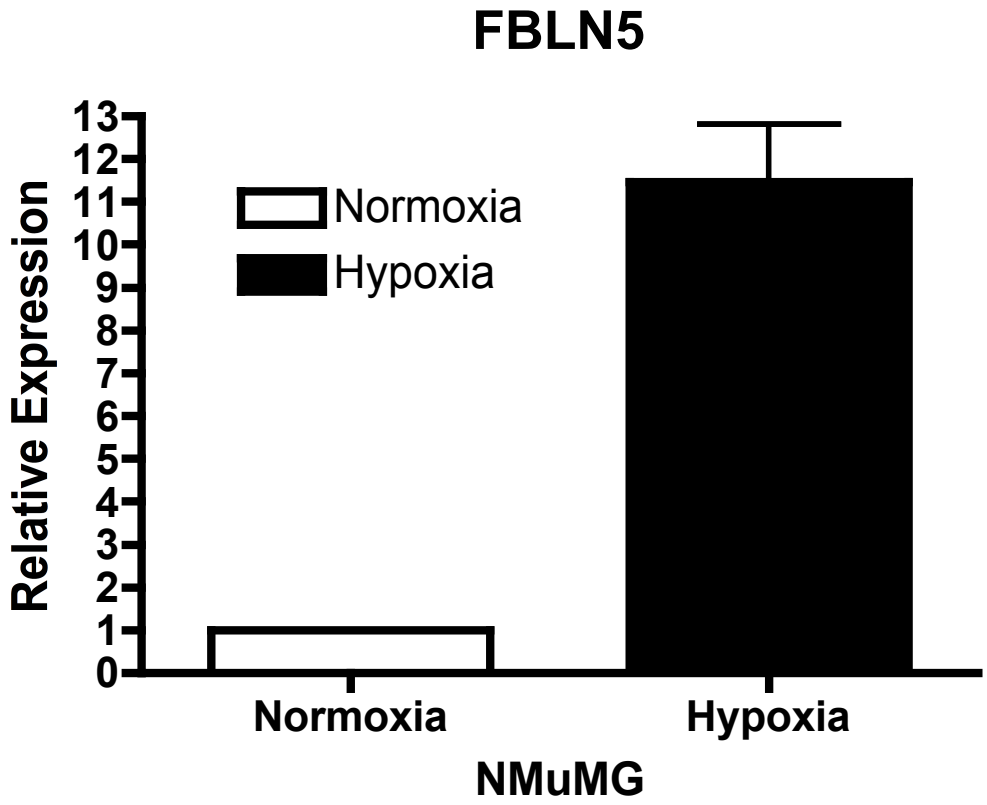
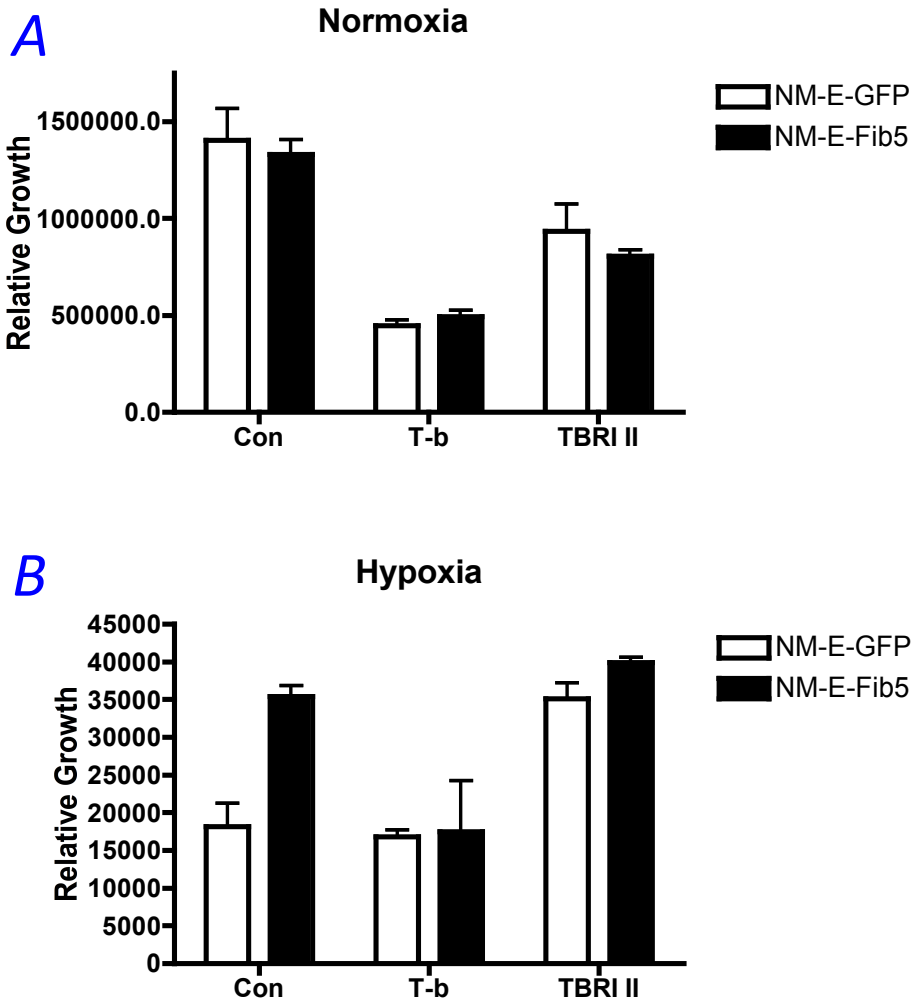


Figure 7

FBLN5 Stimulates the Growth of Malignant NM-E Cells Under Hypoxic Conditions



Legend

Con = Control
T-b = TGF-β
TBRI II = TβRI Inhibitor

NM-E-GFP = GFP-expressing NM-E Cells
NM-E-Fib5 = FBLN5-expressing NM-E Cells

Figure 8

FBLN5 Protects NMuMG Cells from Hypoxia and TGF-β-induced Cell Death

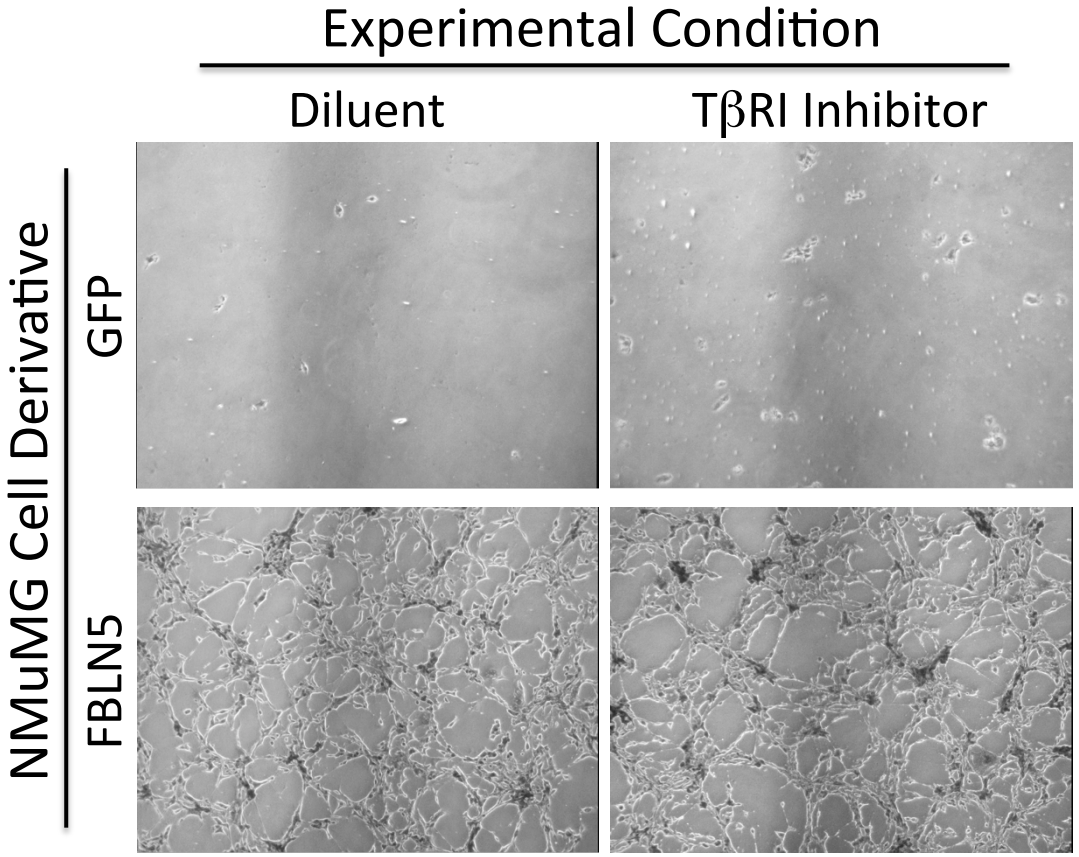


Figure 9

FBLN5 Inhibits Caspase-3 Cleavage and Activation in NMuMG Cells Induced to Undergo Apoptosis in Response to Hypoxia

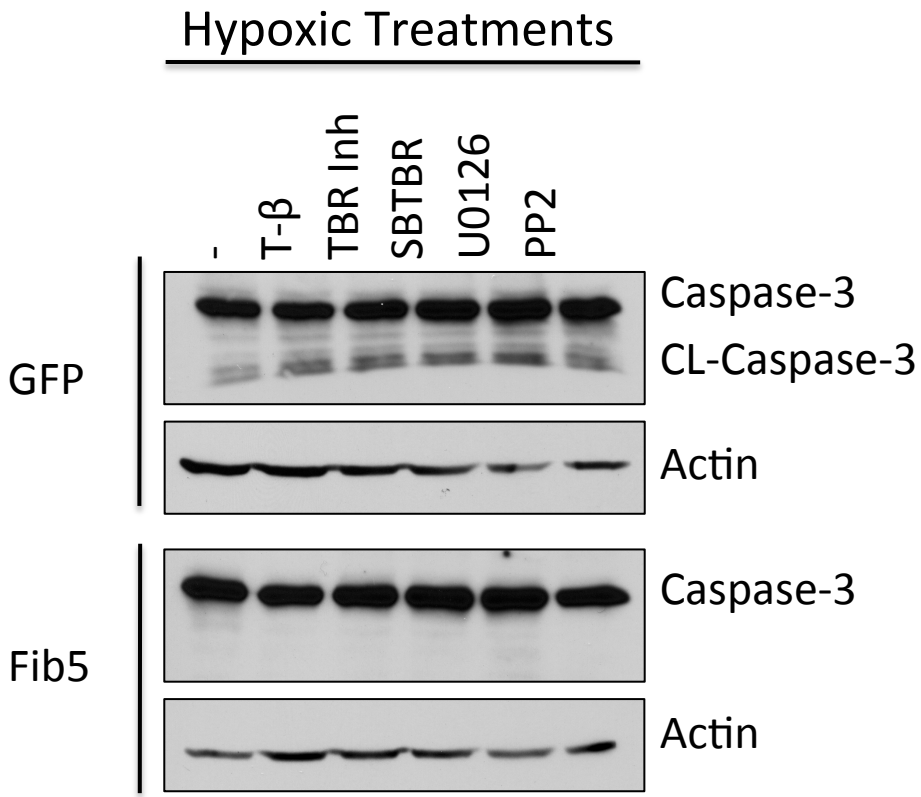
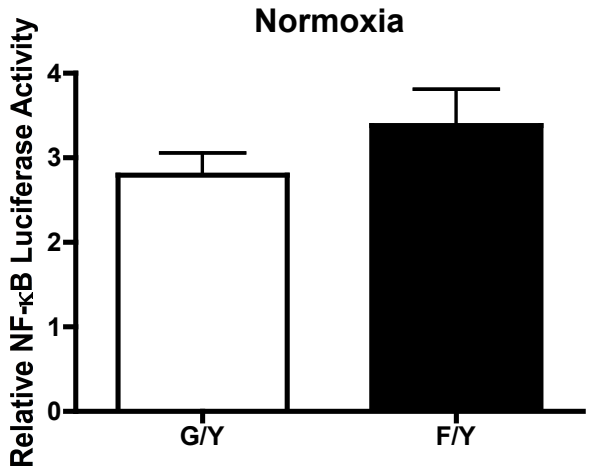


Figure 10

Hypoxia and FBLN5 Cooperate in Activating NF-kB to Suppress Apoptosis of Normal Mammary Epithelial Cells

A



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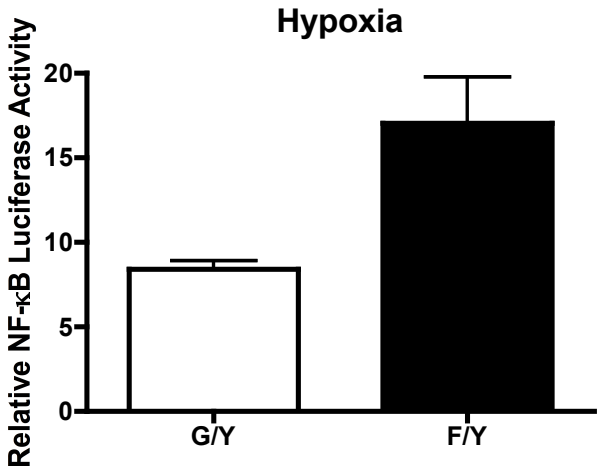


Legend

G = GFP
Y = YFP
F = FBLN5

Figure 11

FBLN5 is a Potent Inducer of MMP Expression in Normal NMuMG Cells

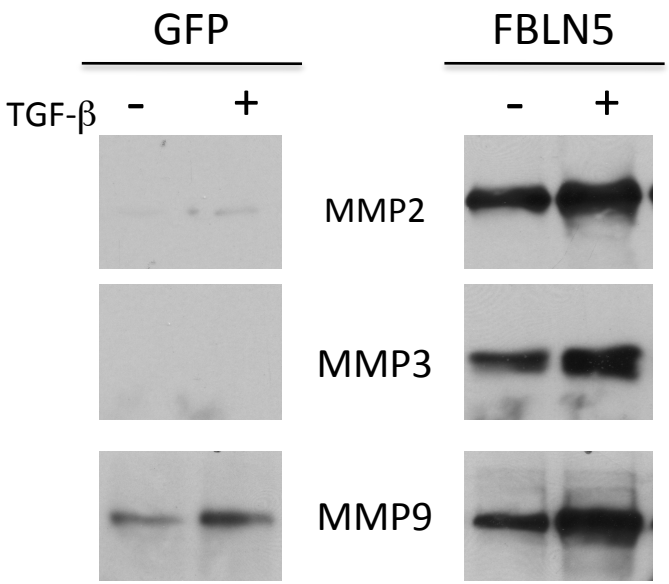


Figure 12

FBLN5 is a Potent Inducer of MMP Expression in Metastatic MECs

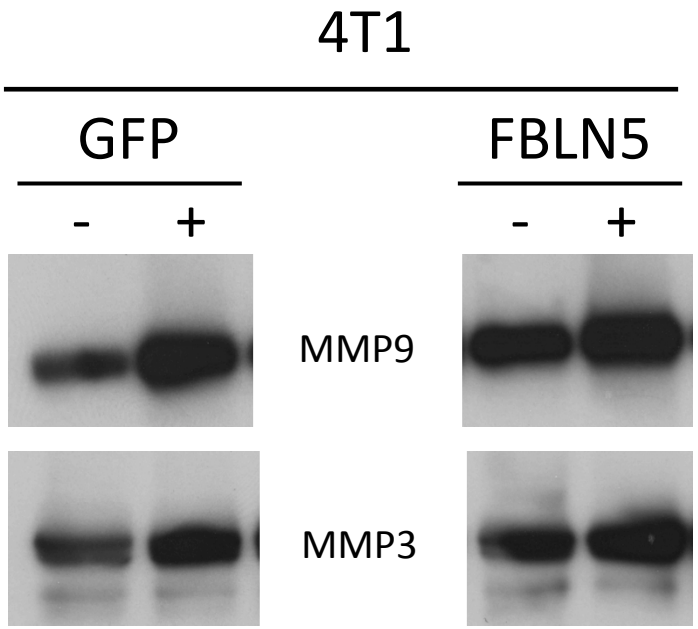
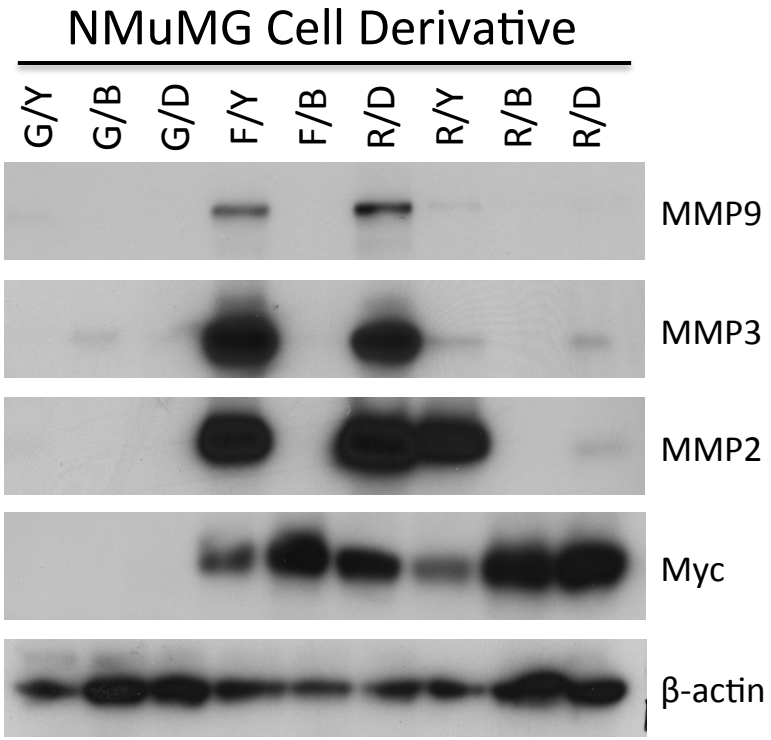


Figure 13

β3 Integrin Masks FBLN5 Stimulation of MMP Expression in NMuMG Cells

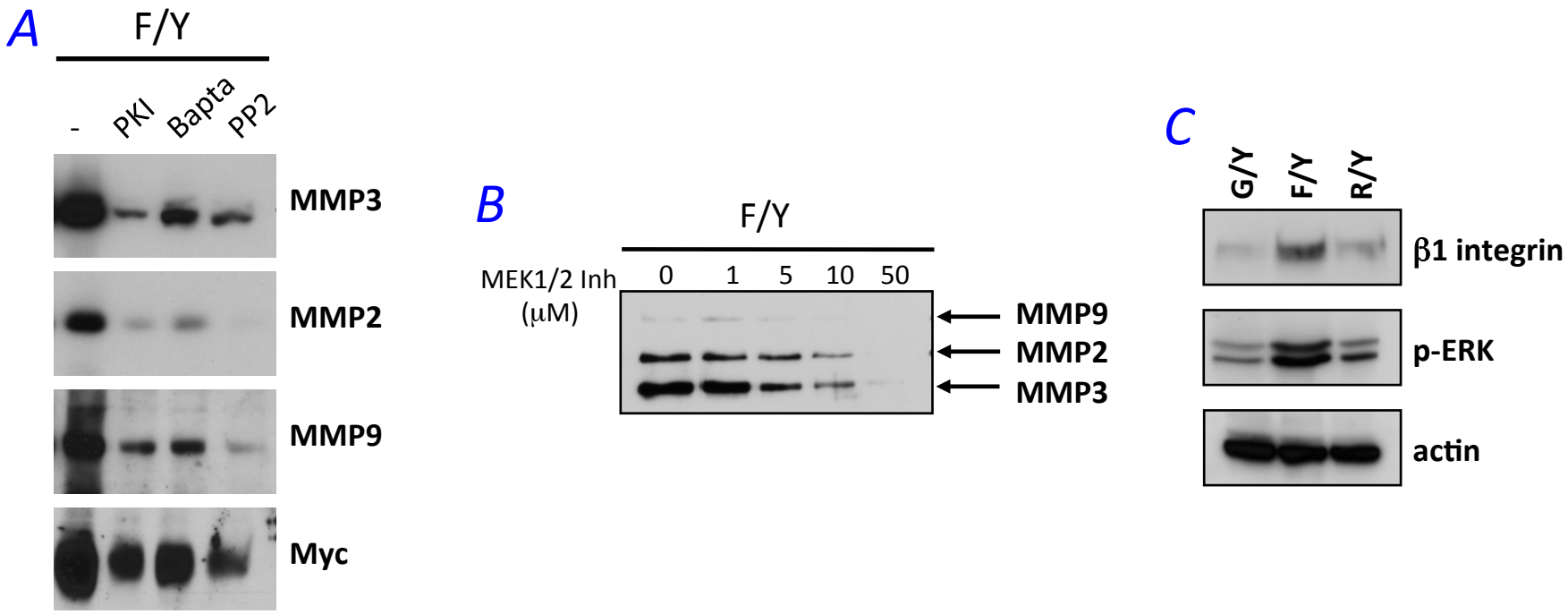


Legend

G = GFP
Y = YFP
F = FBLN5
R = RGE-FBLN5
B = β3 integrin
D = D119A-β3 integrin

Figure 14

FBLN5 Regulates MMP Expression in NMuMG Cells *via* MAP Kinase-, Calcium-, and Src-dependent Mechanisms

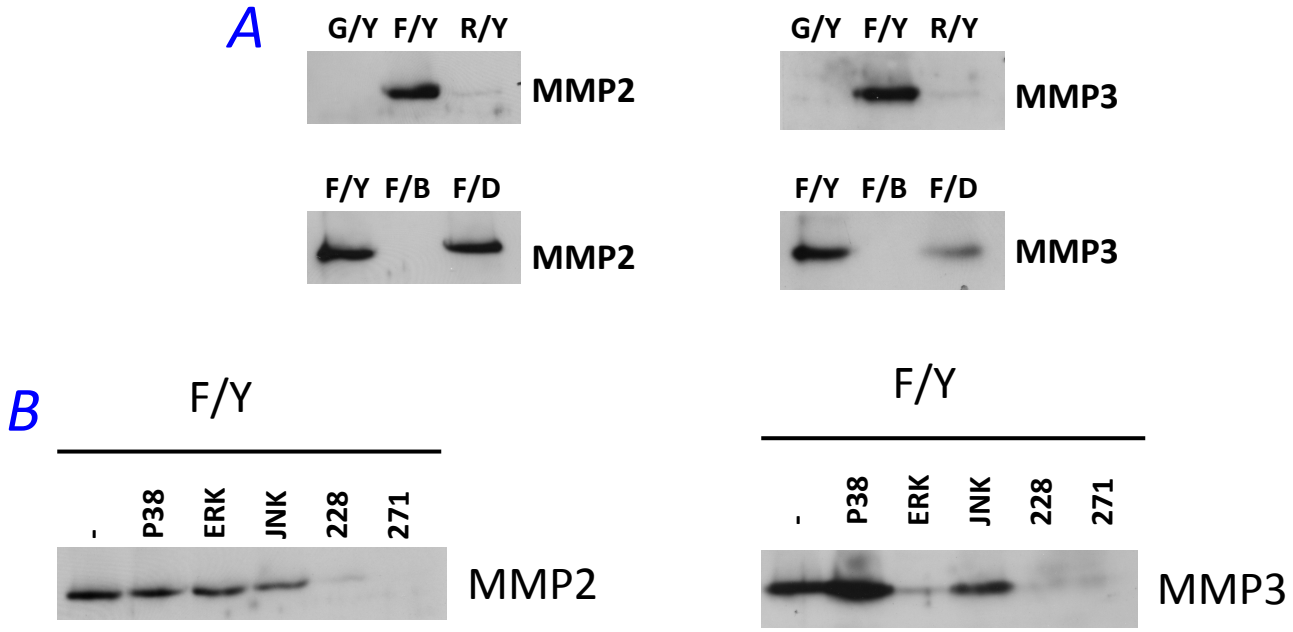


Legend

PKI = mixture of ERK1/2, p38 MAPK, and JNK inhibitors
Bapta = Calcium chealtor
PP2 = Src inhibitor

Figure 15

FBLN5 Induction of MMP Expression is RGD-dependent and Coupled to Activation of ERK and FAK/Pyk2: *Role of β3 Integrin in Suppressing FBLN5 Coupling to MMP Expression*



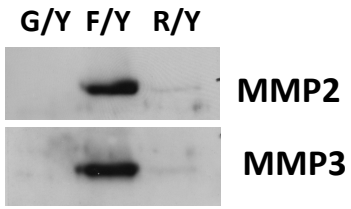
Legend

P38 = p38 MAPK inhibitor	G = GFP
ERK = ERK1/2 inhibitor	Y = YFP
JNK = JNK inhibitor	F = FBLN5
228 = FAK inhibitor	R = RGE-FBLN5
271 = FAK & Pyk2 inhibitor	B = β3 integrin
	D = D119A-β3 integrin

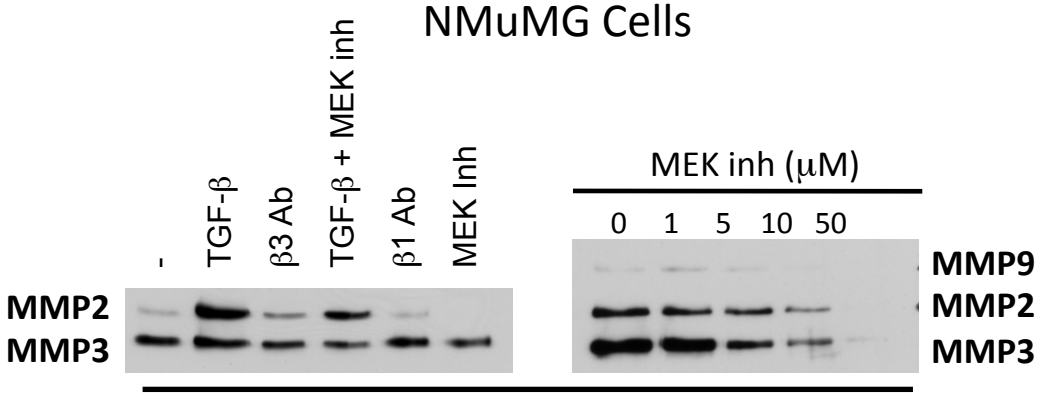
Figure 16

FBLN5 Induces MMP Expression *via* a β1 Integrin-Dependent Pathway in Normal NMuMG Cells

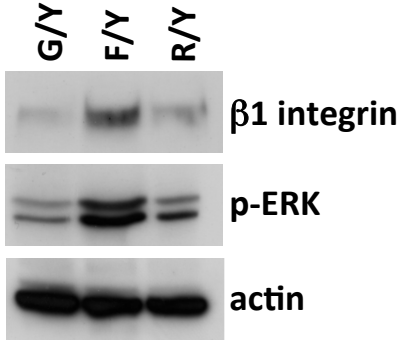
A FBLN5 Requires Integrin Ligation to Induce MMP Expression



B FBLN5 Induces MMP Expression *via* a β1 Integrin & ERK1/2-dependent Pathway in F/Y-Expressing NMuMG Cells



C β1 Integrin Couples FBLN5 to ERK1/2 Activation



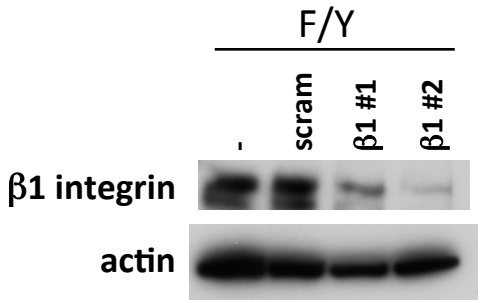
Legend

G = GFP
Y = YFP
F = FBLN5
β3 Ab = Neutralizing β3 integrin antibodies
β1 Ab = Neutralizing β1integrin antibodies
P-ERK = phosphorylated/activated ERK1/2

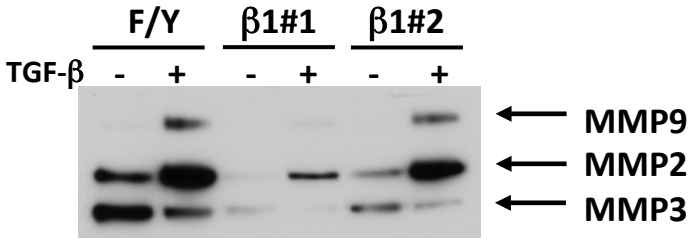
Figure 17

β1 Integrin-depletion Suppresses FBLN5- and TGF-β-mediated MMP Expression in NMuMG Cells

A Monitoring the Extent of β1 Integrin-deficiency in Using Two Independent shRNAs



B β1 Integrin-deficiency Uncouples FBLN5 and TGF-β From MMP Expression



Legend

Y = YFP
F = FBLN5
Scram = nonsilencing shRNA
β1 #1 = β1 integrin shRNA#1
β1 #2 = β1 integrin shRNA#2

Figure 18

FBLN5 Stimulates MMP Expression *via* a EGFR-dependent Pathway

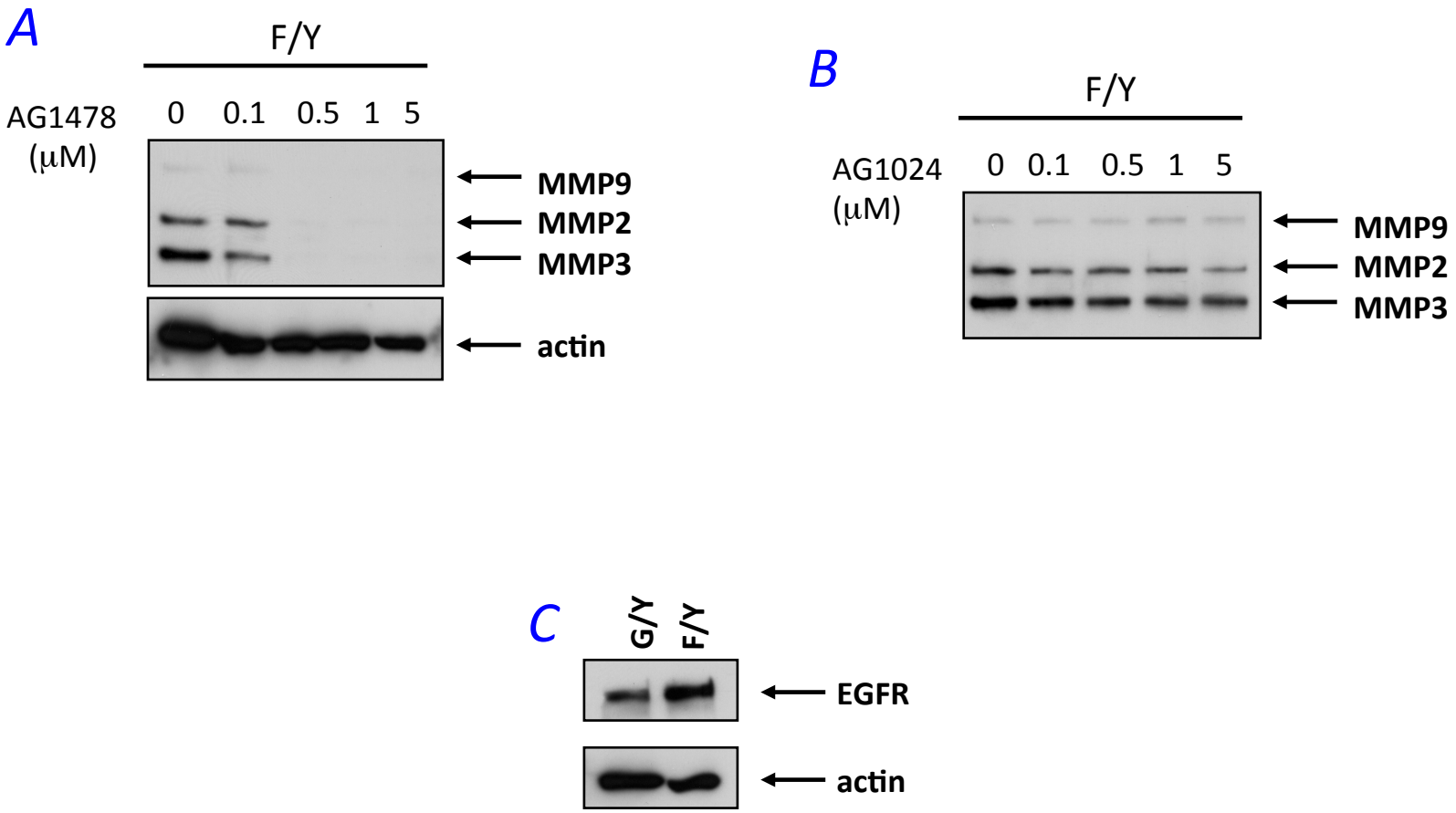


Figure 19

FBLN5 Potentiates EGF-mediated Activation of ERK1/2: *Role of EGFR Stabilization?*

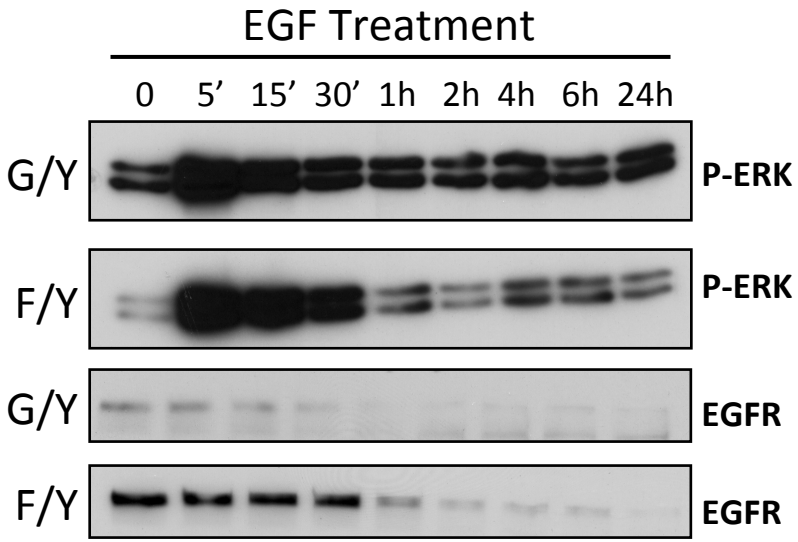


Figure 20

FBLN5 Potentiates MMP Expression and MAP Kinase Mediated Phosphorylation *via* a β1 Integrin- and EGF-dependent Pathway

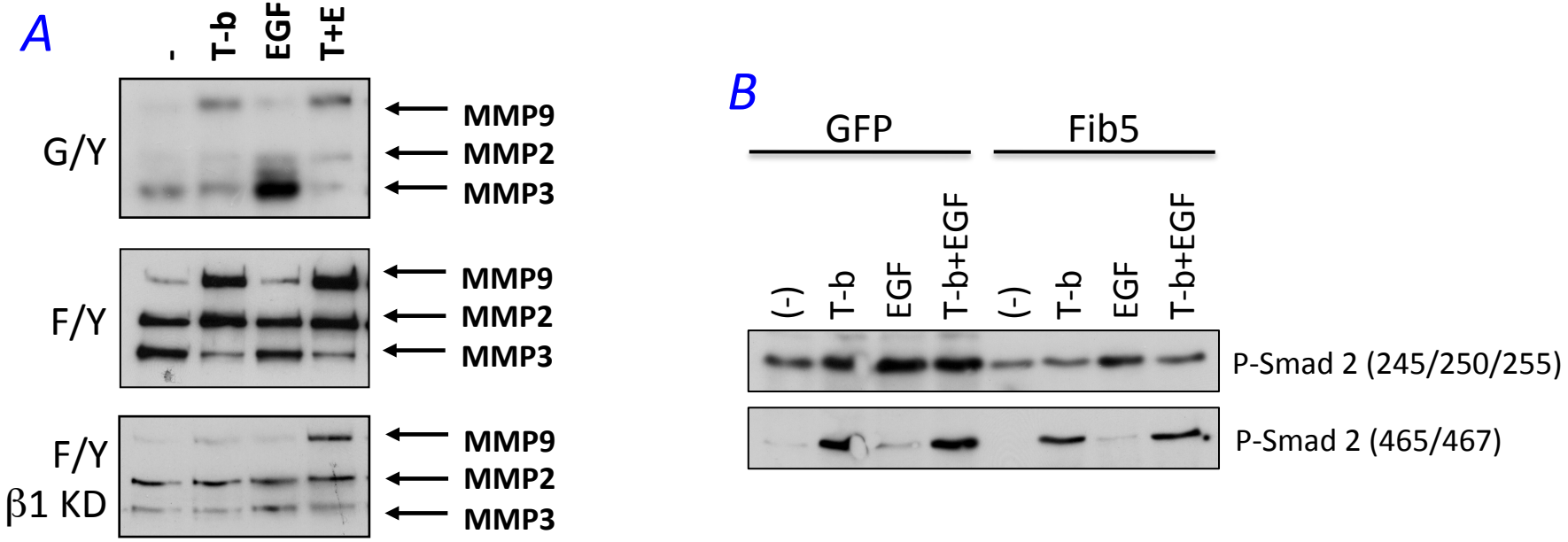
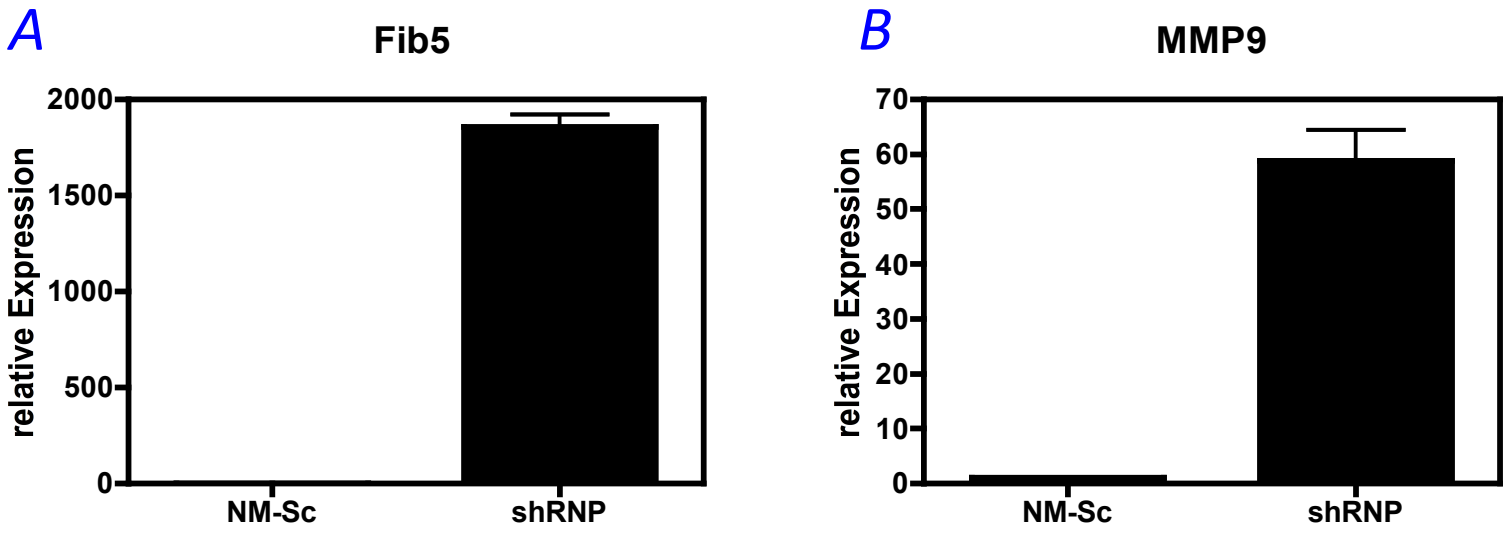


Figure 21

hnRNP E1-Deficiency Dramatically Induces the Expression of FBLN5 and MMP-9 in NMuMG Cells



Legend

NM-Sc = scrambled shRNA
shRNP = shRNA against hnRNP E1

Figure 22

FBLN5 Induces Dab2 Expression *via* a β1 Integrin-dependent Pathway

Legend

G = GFP
Y = YFP
F = FBLN5
shRPE = hnRNP E1 shRNA
sc = nonsilencing shRNA
B1kd = β1 integrin shRNA

Figure 23

FBLN5 Induces EMT *via* a β1 Integrin-dependent Pathway

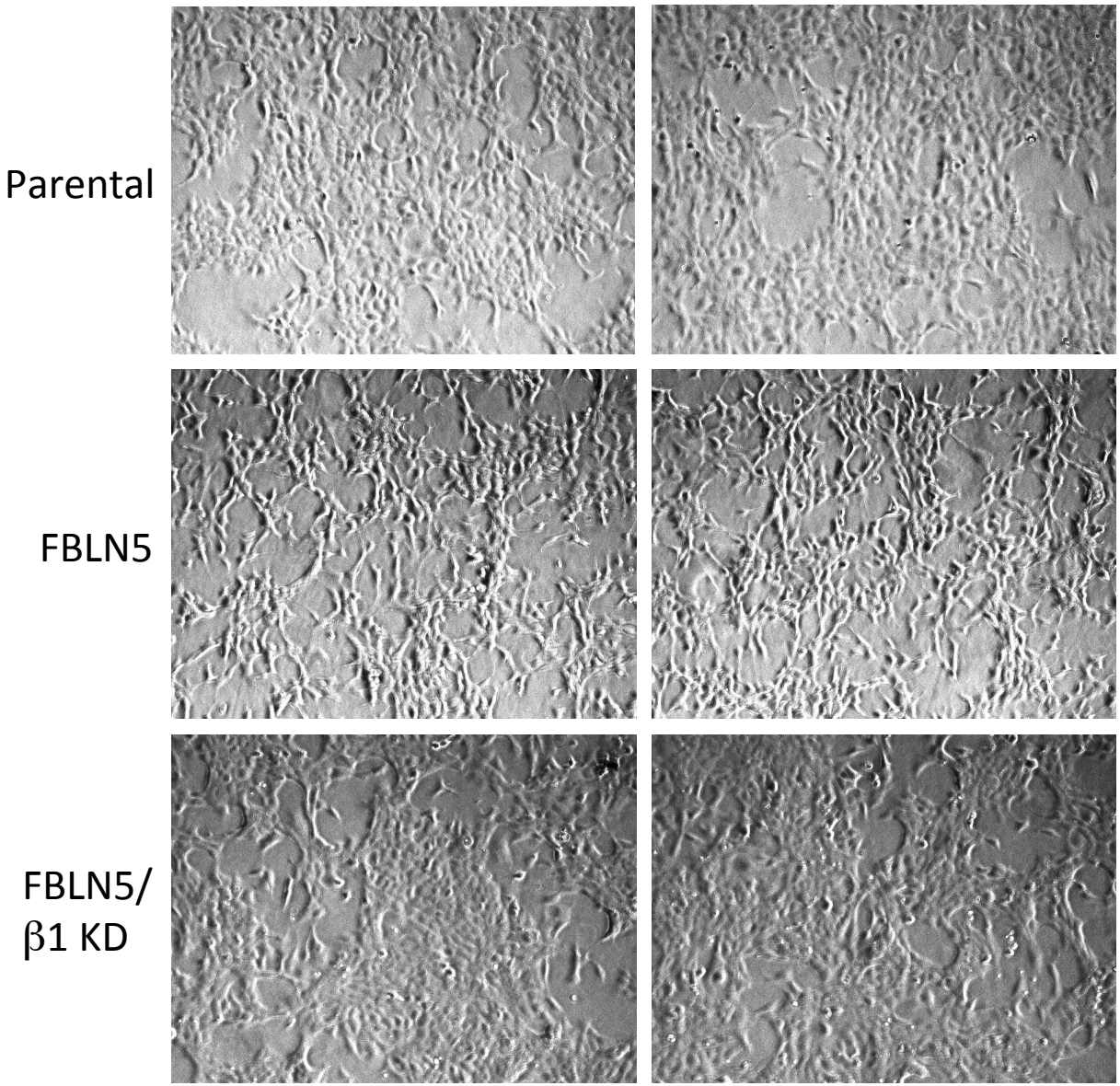


Figure 24

FBLN5 Significantly Enhances EGFR-driven Mammary Tumor Development and Metastatic Progression in Mice