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14. ABSTRACT
Phospholipase C-γ1 (PLC-γ1) mediates cell adhesion and migration through an undefined mechanism. Here, we examine the role of PLC-γ1 in cell-matrix adhesion in a hanging drop assay of cell aggregation. Plcg1 Null (-/-) mouse embryonic fibroblasts formed aggregates that were larger and significantly more resistant to dissociation than cells in which PLC-γ1 is re-expressed (Null + cells). Aggregate formation could be disrupted by inhibition of fibronectin interaction with integrins, indicating that fibronectin assembly may mediate aggregate formation. Fibronectin assembly was mediated by integrin α5β1 in both cell lines, while assays measuring fibronectin assembly revealed increased assembly in the Null cells. Null and Null + cells exhibited equivalent fibronectin mRNA levels and equivalent levels of fibronectin protein in pulse-labeling experiments. However, levels of secreted fibronectin in the conditioned medium were increased in Null cells. The data implicates a negative regulatory role for PLC-γ1 in cell aggregation by controlling the secretion of fibronectin into the media and its assembly into fibrils.

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None provided

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INTRODUCTION

Phospholipase C γ1 (PLC-γ1) is a tyrosine kinase activated enzyme that hydrolyzes phosphotidylinositol 4,5 bisphosphate to form the second messengers inositol 1,4,5 trisphosphate and diacylglycerol. The former stimulates the release of intracellular calcium from the endoplasmic reticulum, while the latter activates protein kinase C. Activation of PLC-γ1 requires tyrosine phosphorylation by receptor or non-receptor tyrosine kinases. PLC-γ1 can be phosphorylated downstream of growth factor stimulation or integrin activation(Wahl *et al.*, 1989a; Wahl *et al.*, 1989b; Kim *et al.*, 1990; Kim *et al.*, 1991; Tvorogov *et al.*, 2005). Adhesion to fibronectin results in Src-mediated phosphorylation and activation of PLC-γ1(Zhang *et al.*, 1999; Tvorogov *et al.*, 2005). Biochemical data and mutagenesis experiments have shown that phosphorylation of Y783 is required for enhancement of PLC-γ1 enzymatic activity (Kim *et al.*, 1991; Sekiya *et al.*, 2004; Tvorogov *et al.*, 2005).

PLC-γ isozymes are required for adhesion, spreading and migration in a variety of cell systems; however, the mechanistic basis of this is unknown (Kundra *et al.*, 1994; Turner *et al.*, 1997; Jones *et al.*, 2005; Tvorogov *et al.*, 2005; Pearce *et al.*, 2007; Wang *et al.*, 2007). PLC-γ1 (-/-) or Null cells exhibit impaired adhesion to fibronectin and this impairment is attenuated by increased concentrations of fibronectin (Tvorogov *et al.*, 2005). PLC-γ1 mediated adhesion to fibronectin requires phosphorylation of PLC-γ1 on Y783. While integrin levels and clustering are not affected by the absence of PLC-γ1, Null fibroblasts exhibit reduced migration towards fibronectin (Tvorogov *et al.*, 2005). PLC-γ1 is known to localize to the leading edge of migrating cells and to cell-matrix adhesions (Todderud *et al.*, 1990; McBride *et al.*, 1991; Plopper *et al.*, 1995).

To further investigate role of PLC-γ1 in adhesion to the extracellular matrix, we have examined fibronectin matrix assembly in *Plcg1* Null and Null + cell lines. The results indicate that PLC-γ1 negatively regulates fibronectin secretion and assembly into fibrils.

BODY

Aim I. Role of PLC-y1 in fibronectin matrix assembly

a. Cell aggregation analysis
b. Examination of Fibronectin assembly
c. Fibronectin mRNA levels
d. Identification of Integrins involved in assembly of Fibronectin
e. Identification of PLC-y1 regulated signaling pathway

Progress- For figures, refer to appendix A

a) Cell aggregation: In my previous reports, I presented data that indicated that Null cells cultured over a day adhere to the substratum tighter. In order to further examine this I performed hanging drop assays in which cells are cultured in a hanging drop over night. This prevents adhesion to the plastic and causes cells to form aggregates as the base of the drop. As expected, Null cells formed tighter aggregates than Null + cells (Figure 1). Cell aggregation could be mediated by cell- cell or cell-matrix interactions (Robinson *et al.*, 2004). As the PLC-γ1 Null and Null + cells are fibroblasts, and fibroblasts secrete and assemble significant amounts of fibronectin, I hypothesized that cell-fibronectin interactions caused the cell aggregations. To test this, I treated the hanging drop aggregates with a 70 kDa fragment of fibronectin that has been shown to inhibit fibronectin assembly (McKeown-Longo and Mosher, 1985). Figure 2 shows that aggregate formation was inhibited by this fragment in both the Null and Null + cells in a dose-dependent manner. At 100 μg/ml of fragment, aggregation was mostly inhibited in the Null + cells while there was little effect in the Null cells. This led to the hypothesis that PLC-γ1 may negatively regulate fibronectin assembly.

b) Fibronectin Assembly: Fibronectin is the most abundant matrix molecule produced by fibroblasts. Once secreted, fibronectin is incorporated into fibrils through interaction with integrins(Mao and Schwarzbauer, 2005). Fibrils can be distinguished from unassembled fibronectin due to insolubility in deoxycholic acid (DOC). Null and Null+ cells were labeled for 4-24 hours with ^{35}S before lysis with a 2% DOC lysis buffer. The insoluble assembled fibronectin was separated from other fibronectin by centrifugation. Fibronectin was immunoprecipitated from each fraction and resolved on a 4-20% SDS-Page. As hypothesized, Null cells assembled more endogenous fibronectin (Figure 3). The increase in fibronectin assembly in Null cells was accompanied by an increase in fibronectin secretion (Figure 3).

c) RNA levels: One explanation for the increase in assembly could be an increase in the amount of fibronectin produced by the cells. In order to examine this, RNA samples from Null and Null + cells were subjected to Northern blot and quantitative RT-PCR for fibronectin mRNA. The levels of fibronectin mRNA were equivalent in Null and Null + cells indicating that PLC-γ1 does not regulate the levels of fibronectin mRNA (Figure 5, Supplementary Figure 2).

Since there in an increase in fibronectin secretion, but not fibronectin mRNA levels, I examine whether PLC-γ1 regulated the transcription of fibronectin by pulsing cells for 10 minutes with ^{35}S-Methionine. At this time point, fibronectin has not been secreted from the cells and comparisons can be made as to the rate of fibronectin secretion in Null and Null + cells. The data in Figure 5C indicates that PLC-γ1 does not regulate fibronectin translation. Since there is an increase in the secretion of fibronectin, I tested whether there was an increase in the total level of proteins secreted by the PLC-γ1 Null cells. Null and Null + cells were labeled with ^{35}S-Methionine for an hour, washed 5 times, and then chased with unlabeled medium for an hour. Conditioned medium was collected and the amount of radiolabeled protein was determined using scintillation counting. The data in Figure 5D indicates that the total protein secretion is equivalent in the *Plcg1* Null and Null + cells.

d) Integrins: Integrins are the receptors for fibronectin. The two main integrins that are involved in fibronectin assembly are $\alpha5\beta1$ and $\alpha V\beta3$. Previous work in the lab showed that the Null and Null + cells have similar integrin expression

profiles(Tvorogov *et al.*, 2005). Integrins α5β1 and αVβ3 are most commonly known to assemble fibronectin in fibroblasts. In order to ensure similar integrin levels and identify which integrins are mediating assembly, both the Null and Null + cell lines were stained for integrin expression and subjected to FACs analysis. As previously seen, integrins α5 and β1 were highly expressed at equal levels while Null cells expressed a 1.5-fold increase in integrin αV (Supplementary Figure 1).

Integrin α5β1 is considered the predominant receptor for fibronectin assembly(Leiss *et al.*, 2008). To determine which integrin pair is responsible for fibronectin assembly in these cells, exogenous assembly assays were performed with cells plated on either fibronectin or vitronectin. If the cells utilize integrin α5β1 to assemble fibronectin, then plating cells on fibronectin would reduce the assembly of exogenous biotinylated fibronectin. However, if cells use integrin αVβ3, also known as the vitronectin receptor, for assembly, then plating cells on vitronectin would also reduce fibronectin assembly. When the Null and Null + cells were plated on fibronectin, there was a significant decrease in fibronectin assembly in both cell lines, while plating on vitronectin had no effect on assembly (Figure 4). This indicates that integrin α5β1 is responsible for fibronectin assembly in Null and Null + cells.

To determine whether integrin α5β1 mediates the cell aggregation observed in Figure 1, Hanging drops were treated with antibodies that inhibit either integrin β1 or β3. Integrin β1 inhibitory antibodies inhibited cell aggregation while integrin β3 inhibitory antibodies had no effect. A combination of both antibodies yielded results similar to the integrin β1 antibody treatment (Figure 4). This indicates that integrin α5β1 mediates fibronectin assembly and cell aggregation in the Null and Null + cells.

e) Potential Mechanisms: The data indicates that PLC-γ1 negatively regulates the selective secretion of fibronectin. Fibronectin secretion by fibroblasts is considered a constitutive process due to the linear increase of secreted fibronectin over time (Figure 3C) (Choi and Hynes, 1979). Little is known about fibronectin secretion in comparison to another fibrous ECM protein, procollagen. Procollagen is a model protein for individualized secretion of large proteins. Procollagen is too large to fit in conventional COPII coated vesicles and is thus transported in larger vesicles from the ER (Fromme and Schekman, 2005). Even though, the COPII components have not

been detected in these vesicles, the machinery is still required for procollagen secretion. In this way, much of the secretion machinery accommodates a vast variety of proteins.

Where could fibronectin secretion be selectively regulated? Once again, the research on procollagen provides precedent for the individualized regulation of protein secretion. Procollagen folding in the ER is mediated by a special molecular chaperone termed Hsp47 (Nakai *et al.*, 1992). In the absence of this protein, procollagen is not secreted and assembled into matrix (Ishida *et al.*, 2006). In excess of this protein, too much procollagen is secreted (Taguchi and Razzaque, 2007).

I propose that PLC-γ1 negatively regulates the level of a fibronectin-specific molecular chaperone. Previous reports show that fibronectin dimerization requires the variable domain on at least one fibronectin molecule in the dimer and that deletion of this domain inhibits fibronectin secretion (Schwarzbauer *et al.*, 1989). The binding of a molecular chaperone to the variable domain of fibronectin may mediate proper folding, dimerization, and secretion of fibronectin as a compact molecule.

The next question that arises is how PLC-γ1 might regulate that chaperone? It is known that PLC-γ1 regulates gene expression through Ca^{2+} release and PKC activation (Nakano *et al.*, 1994; Liao *et al.*, 2001). It is very likely that PLC-γ1 could negatively regulate the expression of a molecular chaperone through the same mechanism.

KEY RESEARCH ACCOMPLISHMENTS

1) PLC-γ1 negatively regulates the secretion of fibronectin
2) Increased fibronectin secretion leads to increased assembly and increased aggregation
3) The function of PLC-γ1 is to prevent the overproduction of fibronectin and the formation of an aberrant matrix

REPORTABLE OUTCOMES

The results of this research have been accepted pending revisions to Experimental Cell Research.

CONCLUSION

While numerous reports have demonstrated a requirement for PLC-γ1 in cell adhesion and/or migration in various cell types (Kundra *et al.*, 1994; Wells and Grandis, 2003; Tvorogov *et al.*, 2005; Shepard *et al.*, 2006; Wang *et al.*, 2007), a mechanistic understanding of this requirement has not been elucidated. The data in this manuscript show that PLC-γ1 has a regulatory role in controlling the amount of fibronectin produced and assembled into fibronectin fibrils. As fibronectin is a major component of the fibroblast extracellular matrix, which mediates cell adhesion and migration, the level of fibronectin production needs to be tightly controlled to avoid an abnormal composition of extracellular matrix. There are several examples of aberrant matrix compositions that affect cell function (George *et al.*, 1993; Sottile *et al.*, 1998; Sottile and Hocking, 2002). For example, when cell migration is measured on increasing concentrations of assembled fibronectin, migration levels exhibit a biphasic effect. Migration rates increase as fibril concentrations increase until an optimal concentration for maximum migration and above this increased concentrations of assembled fibronectin reduce migration (Morla *et al.*, 1994; Hocking and Chang, 2003; Li *et al.*, 2005; Smith *et al.*, 2006).

The data in this manuscript show that while PLC-γ1 does not influence mRNA levels nor production of fibronectin protein within the cell, it does regulate the levels of fibronectin protein that is secreted. This increase in fibronectin secretion is not part of a global effect on secretion as both Null and Null + cell lines secrete equivalent levels of protein. The data also show that the increase in secreted fibronectin in cells genetically deficient in PLC-γ1 is accompanied by an increase in fibronectin assembly into fibrils. The increase in fibronectin assembly is observed in assays that rely on both the assembly of endogenous fibronectin as well as exogenous fibronectin. While the latter might indicate a separate role of PLC-γ1 in assembly, there is published data indicating that an increase in the levels of endogenous fibronectin does in fact result in an increase in the assembly of exogenous fibronectin into fibrils (Bae *et al.*, 2004; Huang *et al.*, 2008). The data would indicate that PLC-γ1 functions to set the limit for the maximal level of fibronectin secretion, and in the absence of this protein is oversecreted.

The fact that the absence of PLC-γ1 increases the level of secreted fibronectin protein, but not mRNA or protein production is novel. The second messengers formed by

PIP_2 hydrolysis are well described for their capacity to effect signaling that impinges on gene expression. However, how these second messengers may affect post-translational processes is less well known.

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Title: PLC-γ1 Regulates Fibronectin Assembly and Cell Aggregation

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Abbreviations: PLC-γ1, phospholipase C γ1; DOC, deoxycholic acid

ABSTRACT

Phospholipase C-γ1 (PLC-γ1) mediates cell adhesion and migration through an undefined mechanism. Here, we examine the role of PLC-γ1 in cell-matrix adhesion in a hanging drop assay of cell aggregation. *Plcg1* Null (-/-) mouse embryonic fibroblasts formed aggregates that were larger and significantly more resistant to dissociation than cells in which PLC-γ1 is re-expressed (Null + cells). Aggregate formation could be disrupted by inhibition of fibronectin interaction with integrins, indicating that fibronectin assembly may mediate aggregate formation. Fibronectin assembly was mediated by integrin α5β1 in both cell lines, while assays measuring fibronectin assembly revealed increased assembly in the Null cells. Null and Null + cells exhibited equivalent fibronectin mRNA levels and equivalent levels of fibronectin protein in pulse-labeling experiments. However, levels of secreted fibronectin in the conditioned medium were increased in Null cells. The data implicates a negative regulatory role for PLC-γ1 in cell aggregation by controlling the secretion of fibronectin into the media and its assembly into fibrils.

INTRODUCTION

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To further investigate role of PLC-γ1 in adhesion to the extracellular matrix, we have examined fibronectin matrix assembly in *Plcg1* Null and Null + cell lines. The results indicate that PLC-γ1 negatively regulates fibronectin secretion and assembly into fibrils.

MATERIALS AND METHODS

Materials: Antibodies for mouse anti-actin, mouse anti-vimentin, and mouse anti-fibronectin were purchased from Sigma Aldrich, while those to integrins α5 (5H10-27), β1 (9EG7), β1 (HA2/5), β3 (2C9.G2) and αV (RMV-7) were purchased from BD Biosciences. The rabbit anti-fibronectin used for immunoprecipitation was purchased from Santa Cruz Biotechnology. Cy3 conjugated goat anti-mouse was purchased from Jackson Immunoresearch and Alexa 647 conjugated goat anti-mouse was a product of Molecular Probes. Mouse and rabbit secondary antibodies used in western blotting were purchased from Licor Biosciences. CycloRGD (RGDfV), and cycloRAD (RADfV) were obtained from Biomol International. Peptides were dissolved at a concentration of 5ug/ml in 25% DMSO/PBS and used at a concentration of 250 μg/ml. The 70 kDa fibronectin fragment (McKeown-Longo and Mosher, 1985) was purchased from Sigma and dissolved in cell culture medium.

Cell Culture and Staining: *Plcg1* Null and Null + immortalized mouse embryonic fibroblasts have been described previously (Ji *et al.*, 1999). Cells were cultured in DMEM supplemented with 10% FBS. In experiments using fibronectin-free FBS, fibronectin was removed by passing FBS through a gelatin-Sepharose column (GE

Health Sciences). For cell staining, cells were fixed in 4% PFA and stained for fibronectin. Stained cells or hanging drop aggregates were visualized using a Zeiss LSM 510 confocal microscope.

Hanging Drop Assay: Null and Null + cells were detached using Accutase (Innovative Cell Technologies) and re-suspended at 500,000 cells/ ml in cell culture medium. The 70 kDa fragment, cyclic RGD or RAD peptides, or integrin β1 or β3 blocking antibodies were added at indicated concentrations. Drops (30 µl) were placed on the lid of a 24 well plate and the lid was inverted over the cell culture wells, which contained PBS to avoid evaporation of the hanging drop. Cells were cultured in the hanging drop overnight. Subsequently, hanging drops were photographed, pipetted 20 times to disrupt cell aggregates and then photographed again using a Leica inverted microscope with 10X objective.

Deoxycholic Acid (DOC) Solubility Assays: The DOC solubility assays have been previously described (Wierzbickka-Patynowski *et al.*, 2004). In brief, 12 $X10^5$ cells were plated in 100 mm dishes in DMEM supplemented with 10% fibronectin-free FBS. After 4-24 hrs, the cells were lysed in 2% DOC lysis buffer (2% DOC, 20mM tris-CL pH 8.8, 2mM PMSF, 2mM EDTA, 2mM iodoacetic acid, and 2 mM N- ethylmaleimide) and lysates were passed through a 25 gauge needle and centrifuged (16,000 x g ,20 min) at 4°C. The supernatant was removed and saved as the DOC soluble fraction, while the pellet was washed in DOC lysis buffer and then re-suspended in 2X LDS reducing sample buffer (Invitrogen). Protein levels were determined for DOC soluble fractions by

BCA assay (Pierce). Equal amounts of DOC-soluble and -insoluble protein were resolved on a 4-12% SDS-PAGE gel.

Metabolic Labeling: Null and Null + cells were placed in labeling medium (methionine and cysteine-free DMEM, 10% FN free FBS, 20 μM unlabeled methionine, and 50 uCi/ml ^{35}S *in vitro* cell labeling mix (GE Healthsciences)). Cells were then harvested as described above and fibronectin was precipitated using mouse anti-human fibronectin (BD Biosciences). To measure levels of secreted fibronectin, conditioned medium was collected and phenylmethylsulfonyl fluoride was added to a final concentration of 2mM. Gelatin-Sepharose beads were added to adsorb fibronectin and the samples were incubated overnight. Beads were washed and 2X reducing sample buffer was added. DOC-soluble, DOC-insoluble, and conditioned medium samples were resolved on a 4-20% SDS-PAGE. The gel was dried and exposed to a Phosphorimager screen. Bands were quantified using Image J software.

For pulse experiments, cells were pulsed for 10 min with ^{35}S-methionine prior to harvesting the cells in TGH lysis buffer (1% Triton X 100, 10% glycerol, 50mM Hepes pH 7.2, 100mM NaCl). Lysates were sonicated and fibronectin was precipitated using rabbit anti-fibronectin. For pulse-chase experiments, cells were labeled for one hr with ^{35}S-methionine prior to a 2-hr chase. Conditioned medium was collected and radioactivity analyzed by scintillation counting. Radioactivity was normalized to protein levels.

Exogenous Assembly Assays: Bovine plasma fibronectin (Sigma) was biotinylated using the manufacturer's protocol (Pierce). Null and Null + cells were re-suspended at a concentration of 1.2 x 10 ^{5} cells/ml in DMEM supplemented with 10% FN-free FBS and

biotinylated fibronectin (20μg/ml) and then plated in 60 mm dishes. After 4 hrs, the cells were lysed and DOC-soluble and -insoluble fractions were isolated. Fractions were resolved on 4-12% SDS-PAGE gels and transferred to a PVDF membrane. Membranes were blotted with NeutrAvidin® HRP (Pierce) or Streptavidin IR680 (Licor inc) and visualized by chemiluminescence or an Oddysey fluorescence imager (Licor inc).

RNA Analysis: Cells were plated at equal density and cultured overnight. The next day, RNA was isolated using the Qiagen RNeasy mini kit. Total RNA was subjected to northern blot and QRT-PCR. For northern blots, the 683 bp human fibronectin probe was cut from plasmid pSP73 RFN 2375-6090 (provided by Dr. Jean Schwarzbauer, Princeton University) using EcoRV and BamHI. Blots were stripped and re-probed for cyclophilin as a loading control. Fibronectin QRT-PCR primers were purchased from Applied Biosystems. cDNA was generated using the iscript cDNA Synthesis Kit (BioRad). QRT-PCR was performed using IQ Supermix (BioRad) and the Biorad ICycler. Values were normalized to actin using the ΔΔcT method.

RESULTS

PLC-γ1 Deficiency Increases Cell Aggregation: *Plcg1* Null and Null + cells were cultured in hanging drops overnight to examine cell aggregation. The data in Figure 1A demonstrate that the Null cells formed a single compact aggregate whereas aggregates formed by Null + cells were smaller and more numerous. Attempts to disperse Null cell aggregates by pipetting were unsuccessful, while similar attempts with Null + cells yielded a significant number of single cells or doublets (Figure 1B). These results show that cells deficient in PLC-γ1 display increased aggregation under these culture conditions.

Cell aggregation is mediated by either cadherin -dependent cell:cell interactions and/or fibronectin-dependent cell:matrix interactions (Lash *et al.*, 1984; Robinson *et al.*, 2004). Fibronectin is the major matrix protein secreted by fibroblasts and following secretion, fibronectin is assembled into fibrils by integrins α5β1 or αVβ3 (Mao and Schwarzbauer, 2005). To determine whether the cell aggregates observed in Figure 1 contain assembled fibronectin, aggregates were fixed and stained for fibronectin. Fibronectin was detected in the aggregates of both Null and Null + cells (data not shown). Western blotting showed that Null and Null + cells express equivalent levels of N-cadherin (data not shown).

To determine whether fibronectin assembly contributes to the cell aggregation shown in the hanging drops of Figure 1, cells were treated with cyclic RGD or inactive cyclic RAD to block fibronectin interaction with the integrins. Treatment with RGD peptides has been shown to inhibit fibronectin assembly, as the RGD sequence of

fibronectin is a critical motif for recognition by integrins (Pierschbacher and Ruoslahti, 1984; Lash *et al.*, 1987; Nagai *et al.*, 1991; Sechler *et al.*, 1997; Robinson *et al.*, 2004; Feral *et al.*, 2007). However, treatment with cyclic RGD had no effect on the increased cell aggregation exhibited by Null cells (data not shown). Cyclic RGD treated aggregates stained positive for fibronectin, indicating that the peptide was ineffective at blocking fibronectin interaction with the cell.

Since previous reports have indicated that RGD peptides are not stable during incubations longer than 6-8 hrs and that cyclic RGD may be more specific for integrins αVβ3 than α5β1(Lash *et al.*, 1987; Pfaff *et al.*, 1994), a 70 kDa N-terminal fibronectin fragment, generated from proteolytic digestion of fibronectin, was used (McKeown-Longo and Mosher, 1985; Lash *et al.*, 1987; McDonald *et al.*, 1987; Pfaff *et al.*, 1994). The region of fibronectin encompassed by this fragment has been shown to block initiation of fibronectin assembly and also to bind integrin α5β1 (McKeown-Longo and Mosher, 1985; Takahashi *et al.*, 2007). Treatment of hanging drops with the 70 kDa fragment completely abolished cell aggregation at concentrations at or above 500 μg/ml. At a concentration of 100 μg/ml, cell aggregation was decreased by this fragment in the Null + cells, but had no effect on the Null cell aggregation (Figure 2A).

Influence of PLC-γ1 on Fibronectin Assembly: To determine whether fibronectin assembly is increased in Null cells, deoxycholic acid (DOC) assembly assays were performed. Null and Null + cells were labeled with ^{35}S methionine to compare the assembly of endogenous fibronectin. The results, shown in Figure 3, demonstrate that the Null cells assemble more fibronectin than Null + cells. An increase in the level of labeled

fibronectin in the Null cell conditioned medium was also detected. Increased fibronectin assembly was also observed in the Null cells when the assembly of exogenous fibronectin was measured (Figure 4). These results show that the increased cell aggregation observed with Null cells is correlated with the presence of fibronectin fibrils in the aggregates and increased fibronectin assembly.

Increased fibronectin assembly can reflect increased integrin or fibronectin expression. Previous work with these cells revealed similar integrin expression in the two cell lines (Tvorogov *et al.*, 2005). To confirm this, we compared the expression of integrins α5, αV, and β1 in Null and Null + cells by FACS analysis. Null cells express approximately 1.25-fold more integrin α5, 1.2-fold more β1, and 1.4-fold more αV as compared to Null + cells (Supplementary Figure 1). To determine which integrin pair is responsible for fibronectin assembly in these cells, exogenous assembly assays were performed with cells plated on either fibronectin or vitronectin. If the cells utilize integrin α5β1 to assemble fibronectin, then plating cells on fibronectin would reduce the assembly of exogenous biotinylated fibronectin. However, if cells use integrin αVβ3, also known as the vitronectin receptor, for assembly, then plating cells on vitronectin would also reduce fibronectin assembly. When the Null and Null + cells were plated on fibronectin, there was a significant decrease in fibronectin assembly in both cell lines, while plating on vitronectin had no effect on assembly (Figure 4). This indicates that integrin α5β1 is responsible for fibronectin assembly in Null and Null + cells.

To test whether the α5β1 integrin is required for the cell aggregation observed in Figure 1, blocking antibodies to either integrin β1 or β3 (as a control) were incorporated into the hanging drop assay (Figure 4B). Integrin β1 blocking antibodies effectively

reduced cell aggregation, while integrin β3 antibodies had no effect. Treatment with both antibodies yielded similar results to integrin β1 antibody treatment, indicating that the β1, but not β3 integrin is required for the formation of tight aggregates in Null and Null + cells.

PLC-γ1 Negatively Regulates the Levels of Secreted Fibronectin: As PLC-γ1 Null cells display an increased capacity to assemble fibronectin into fibrils, it is possible that this reflects increased levels of fibronectin mRNA and/ or protein. To determine whether steady-state fibronectin mRNA levels differ in the two cell lines, RNA was extracted from Null and Null + cells and mRNA levels were compared using qRT-PCR. The data show that steady state fibronectin mRNA levels are equivalent in both cell lines using both qRT-PCR (Figure 5A) and northern blotting (Supplementary Figure 2).

DOC assembly assays indicate that there may be an increase in fibronectin protein expression in the conditioned medium of Null cells (Figure 3). Therefore, fibronectin levels in the conditioned medium of Null and Null + cells were compared. Consistent with previous data, these results show that the Null cells secrete two-fold more fibronectin into the medium than Null + cells (Figure 5B). To determine whether fibronectin translation is increased in Null cells, Null and Null + cells were pulsed with ^{35}S-methionine for 10 min prior to lysis and fibronectin immunoprecipitated. The results, shown in Figure 5, show that fibronectin protein production is equivalent in Null and Null + cells.

To determine whether PLC-γ1 exerts a global effect on protein secretion, a pulse chase experiment was performed. Cells were labeled with ^{35}S-methionine for 90 min followed by a 90 min chase. Conditioned medium was collected and the radioactivity quantitated by scintillation counting. Null and Null + cells secreted equivalent levels of radiolabeled protein, indicating that PLC-γ1 selectively regulates the secretion of fibronectin (Figure 5D).

DISCUSSION

While numerous reports have demonstrated a requirement for PLC-γ1 in cell adhesion and/or migration in various cell types (Kundra *et al.*, 1994; Wells and Grandis, 2003; Tvorogov *et al.*, 2005; Shepard *et al.*, 2006; Wang *et al.*, 2007), a mechanistic understanding of this requirement has not been elucidated. The data in this manuscript show that PLC-γ1 has a regulatory role in controlling the amount of fibronectin produced and assembled into fibronectin fibrils. As fibronectin is a major component of the fibroblast extracellular matrix, which mediates cell adhesion and migration, the level of fibronectin production needs to be tightly controlled to avoid an abnormal composition of extracellular matrix. There are several examples of aberrant matrix compositions that affect cell function (George *et al.*, 1993; Sottile *et al.*, 1998; Sottile and Hocking, 2002). For example, when cell migration is measured on increasing concentrations of assembled fibronectin, migration levels exhibit a biphasic effect. Migration rates increase as fibril concentrations increase until an optimal concentration for maximum migration and above

this increased concentrations of assembled fibronectin reduce migration (Morla *et al.*, 1994; Hocking and Chang, 2003; Li *et al.*, 2005; Smith *et al.*, 2006).

The data in this manuscript show that while PLC-γ1 does not influence mRNA levels nor production of fibronectin protein within the cell, it does regulate the levels of fibronectin protein that is secreted. This increase in fibronectin secretion is not part of a global effect on secretion as both Null and Null + cell lines secrete equivalent levels of protein. The data also show that the increase in secreted fibronectin in cells genetically deficient in PLC-γ1 is accompanied by an increase in fibronectin assembly into fibrils. The increase in fibronectin assembly is observed in assays that rely on both the assembly of endogenous fibronectin as well as exogenous fibronectin. While the latter might indicate a separate role of PLC-γ1 in assembly, there is published data indicating that an increase in the levels of endogenous fibronectin does in fact result in an increase in the assembly of exogenous fibronectin into fibrils (Bae *et al.*, 2004; Huang *et al.*, 2008). The data would indicate that PLC-γ1 functions to set the limit for the maximal level of fibronectin secretion, and in the absence of this protein is oversecreted

The fact that the absence of PLC-γ1 increases the level of secreted fibronectin protein, but not mRNA or protein production is novel. The second messengers formed by PIP_2 hydrolysis are well described for their capacity to effect signaling that impinges on gene expression. However, how these second messengers may affect post-translational processes is less well known.

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FIGURE LEGENDS

Figure 1. Assessment of cell aggregation by Null and Null + cells: A) Cells were detached and 5 X 10^5 cells were re-suspended in cell culture medium. 30 μ1 drops were added to the lid of a 24 well dish and hanging drops were cultured overnight. The next morning, drops were photographed (left panels), pipetted 20 times to disperse, and photographed again (middle panels). Pictures are representative images of multiple hanging drops. Cellular aggregates were stained for fibronectin and then visualized using a Zeiss confocal microscope (right panels). B) Quantification of the number of singlet's

and doublets in photographs before and after pipetting. Values are the average of 5 different hanging drops. Error bars indicate standard deviation.

Figure 2. Treatment of Hanging Drops with 70 kDa fragment: Null and Null + hanging drops were treated as in Figures 1 and 2 except for the addition of 1000 μg/ml, 500 μg/ml, or 100 ug/ml 70 kDa fibronectin fragment.

Figure 3. Assessment of fibronectin assembly in Null and Null + cells: Null and Null + cells were metabolically labeled with ^{35}S methionine for the times indicated. Parts A, B, and C: Top panels - phosphorimager image. Arrows indicate 220 kDa fibronectin; Bottom panels- densitometric analysis. Values are relative to Null cells at 24 hours. Gray bars represent Null cells and white bars represent Null + cells. A) DOC-insoluble fraction; B) DOC-soluble fraction; C) Conditioned media; D) Total cell lysate.

Figure 4. Fibronectin assembly is mediated by Integrin α5β1 in Null and Null + cells: A) Exogenous assembly assay; Cells were plated on 10ug/ml fibronectin or 5 ug/ml vitronectin in fibronectin free medium prior to the addition of biotinylated fibronectin. Cells were incubated overnight followed by collection of lysates and separation into DOC-soluble and insoluble fractions. Right panels: DOC-insoluble fractions. Biotinylated fibronectin is detected by blotting with IR Dye 680 Streptavidin. Vimentin serves as a loading control. Left Panels: DOC-soluble fraction: Actin serves as a loading control. B) Densitometric analysis of DOC insoluble fraction in panel A. Gray bars represent Null cells. White bars represent Null + cells. C) Hanging drop assay: Cells were

treated as in figures 1 and 2 except for treatment with 100 μg/ml inhibitory antibodies against integrins β1 (HA 2/5), β3 (2C9.G2) or a combination of both.

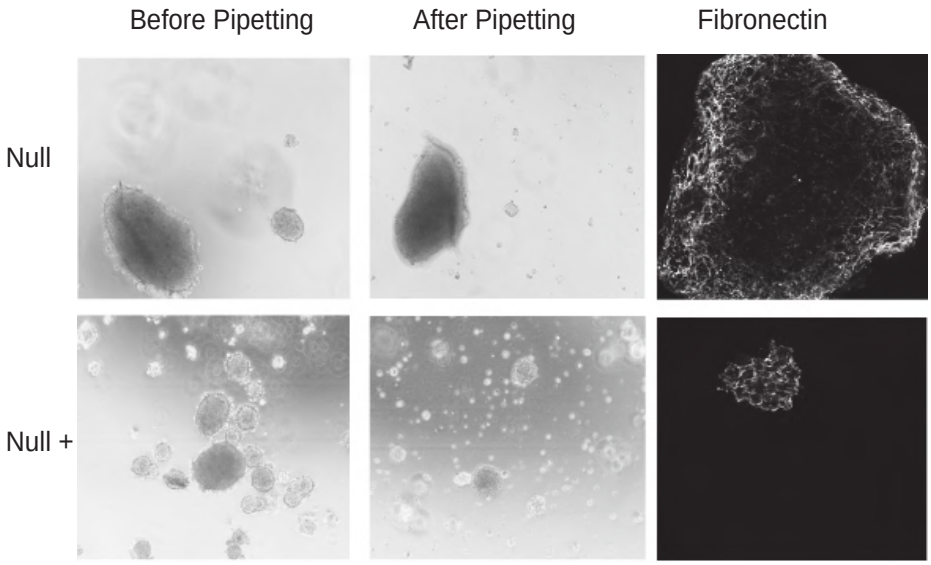
Figure 5. Comparison of fibronectin mRNA and protein levels: A) QRT-PCR of RNA extracted from Null and Null + cells. B) Null and Null + cells were plated and cultured overnight. Cell lystates were harvested in RIPA buffer and conditioned medium (CM) was collected. Equal amounts of protein were resolved by SDS-PAGE followed by detection with rabbit FN and mouse actin antibodies. C) Cells were pulsed for 10 minutes with ^{35}S methionine followed by lysis and Immunoprecipitation for fibronectin. Abbreviations FN Fibronectin; TCL total cell lysate D) Null and Null + were pulsed for ^{35}S methionine followed by a two hour chase. The levels of radioactivity were determined using scintillation counter and normalized to cell lysate protein levels.

Supplementary Figure 1. Integrin surface expression in Null and Null + cells: A) Facs analysis of Null and Null+ cells stained for mouse integrins α5, β1, and αV. B) Statistical data from Facs analysis.

Supplementary Figure 2. Northern hybridization using a fibronectin probe. RNA isolated from Null and Null + cells subjected to northern hybridization using a probe for fibronectin. The membrane was stripped and re-blotted for cyclophilin as a loading control

Figure 1

A.


Before Pipetting
After Pipetting
Fibronectin
Null
Null +

B.

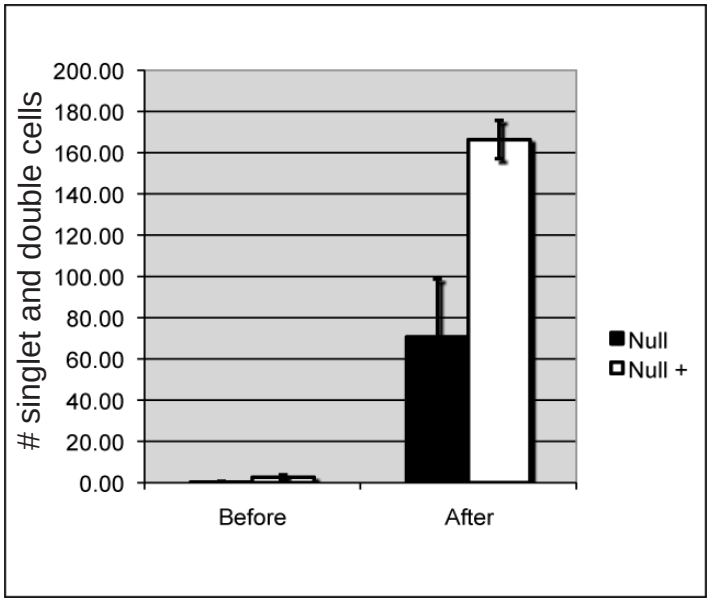

singlet and double cells
200.00
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Before
After
Null
Null +

Figure 2

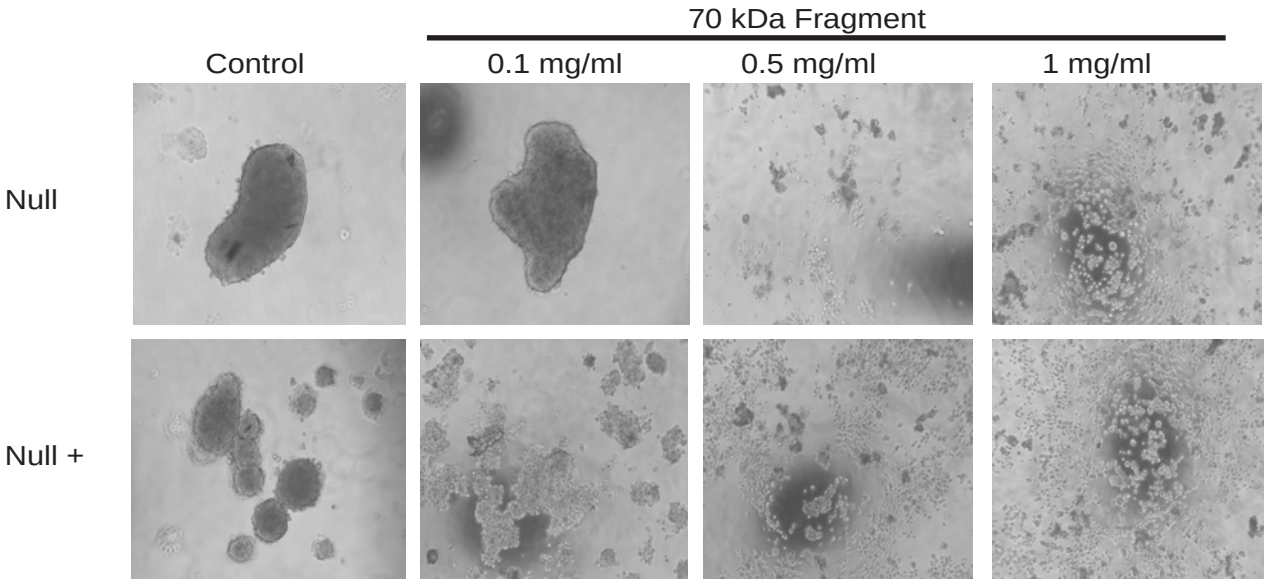


Figure 3

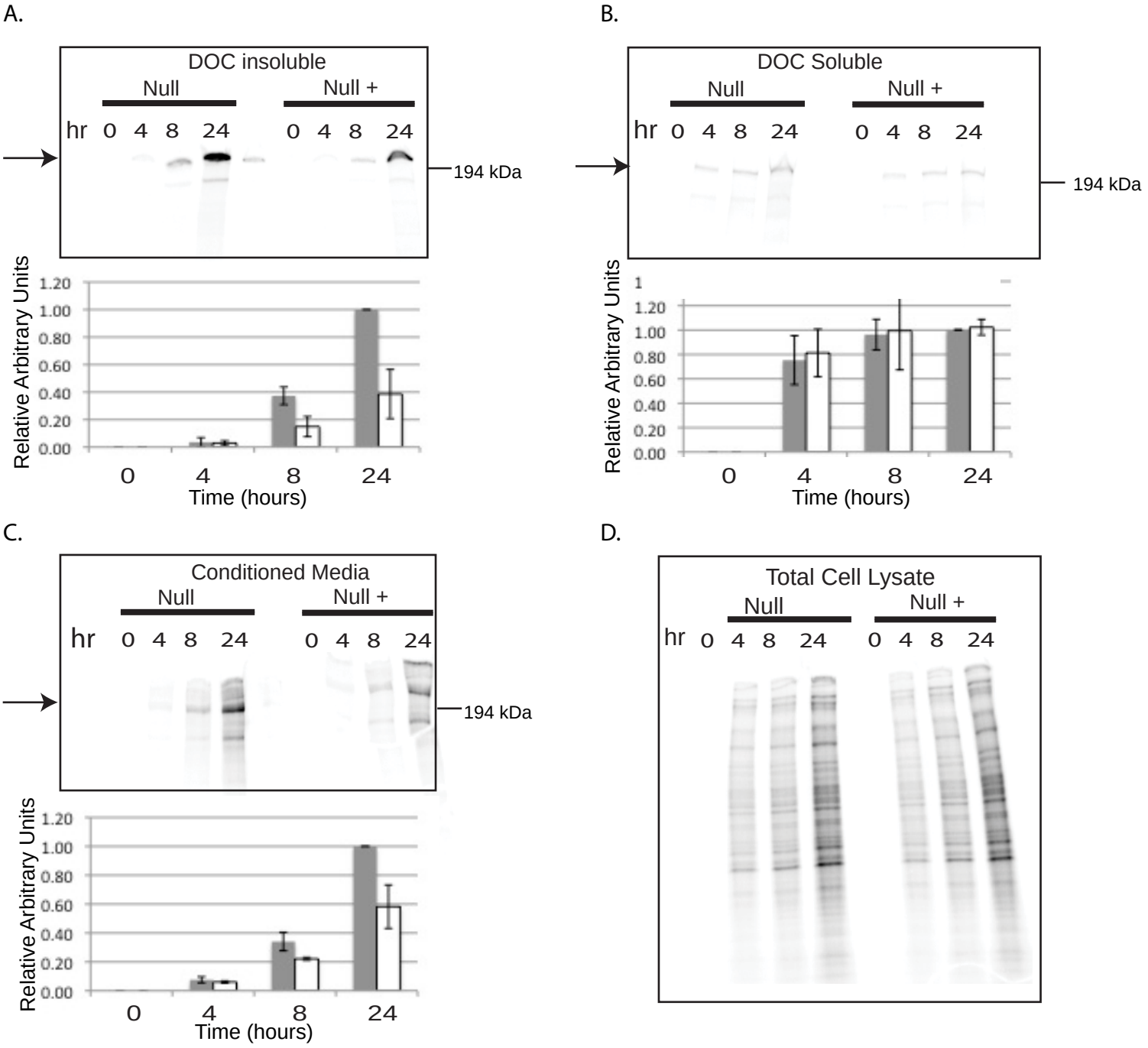
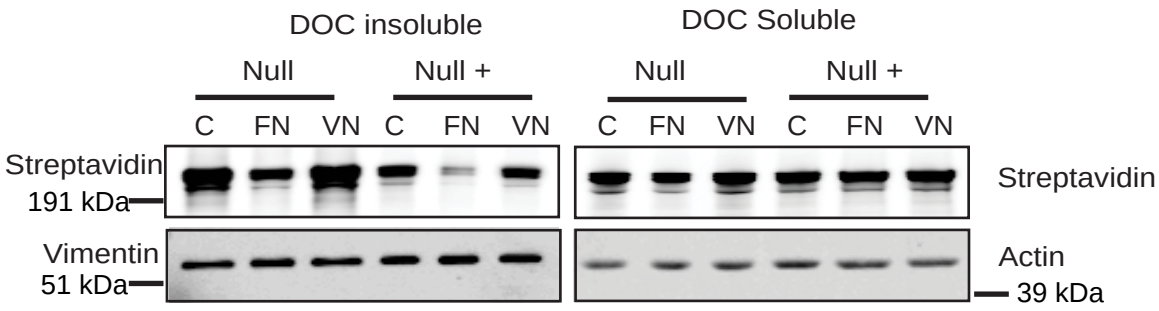

A.
DOC insoluble
Null
Null +
hr 0 4 8 24 0 4 8 24
194 kDa
Relative Arbitrary Units
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0 4 8 24
Time (hours)
B.
DOC Soluble
Null
Null +
hr 0 4 8 24 0 4 8 24
194 kDa
Relative Arbitrary Units
1
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0 4 8 24
Time (hours)
C.
Conditioned Media
Null
Null +
hr 0 4 8 24 0 4 8 24
194 kDa
Relative Arbitrary Units
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0 4 8 24
Time (hours)
D.
Total Cell Lysate
Null
Null +
hr 0 4 8 24 0 4 8 24

Figure 4

A.


DOC insoluble
DOC Soluble
Null
Null +
Null
Null +
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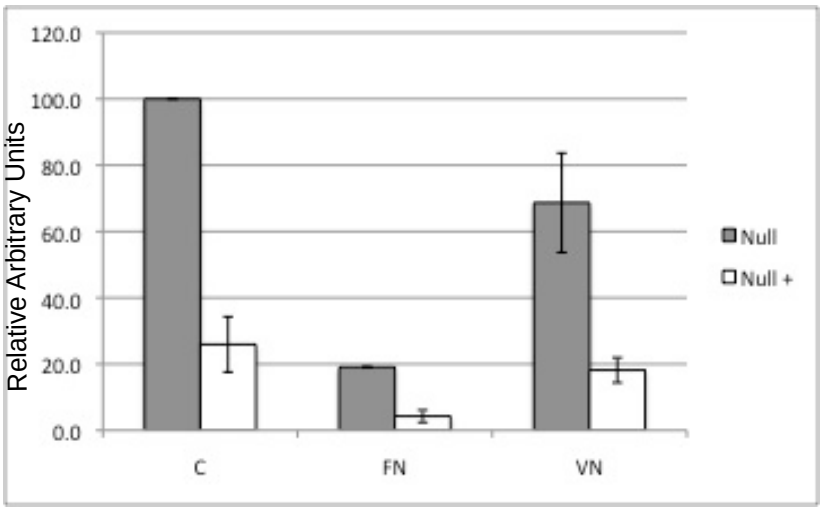
Streptavidin
191 kDa
Vimentin
51 kDa
Streptavidin
Actin
39 kDa

B.


120.0
100.0
80.0
60.0
40.0
20.0
0.0
Relative Arbitrary Units
C
FN
VN
Null
Null +

C.

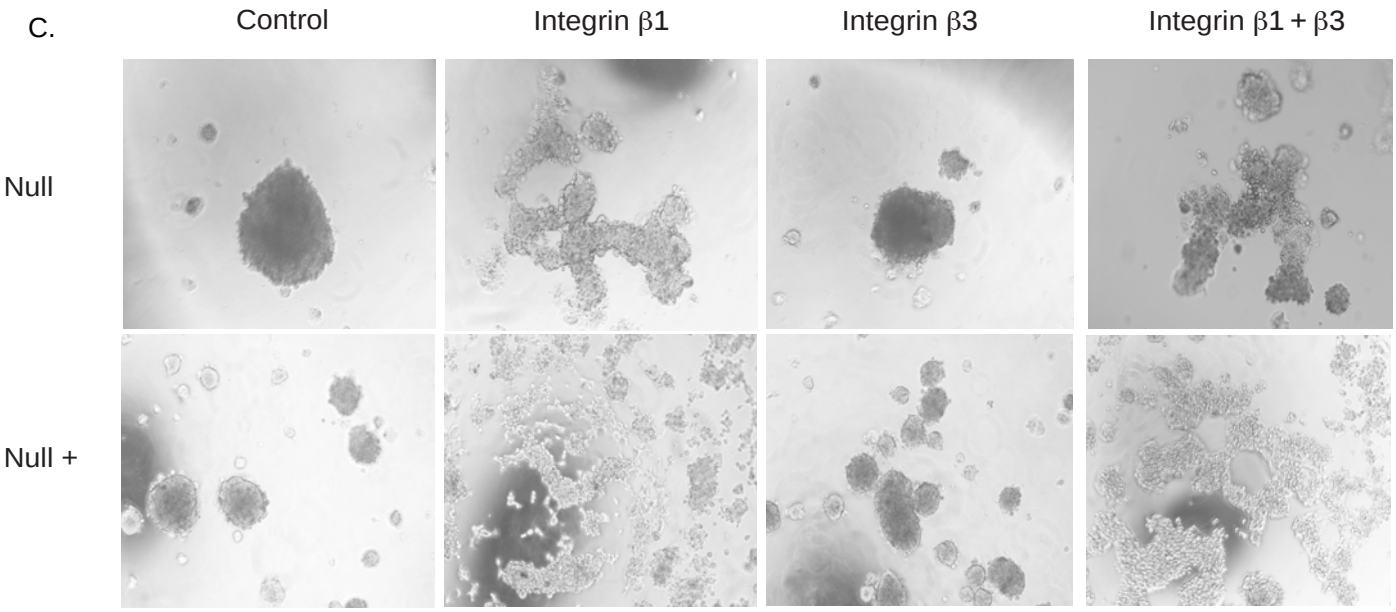
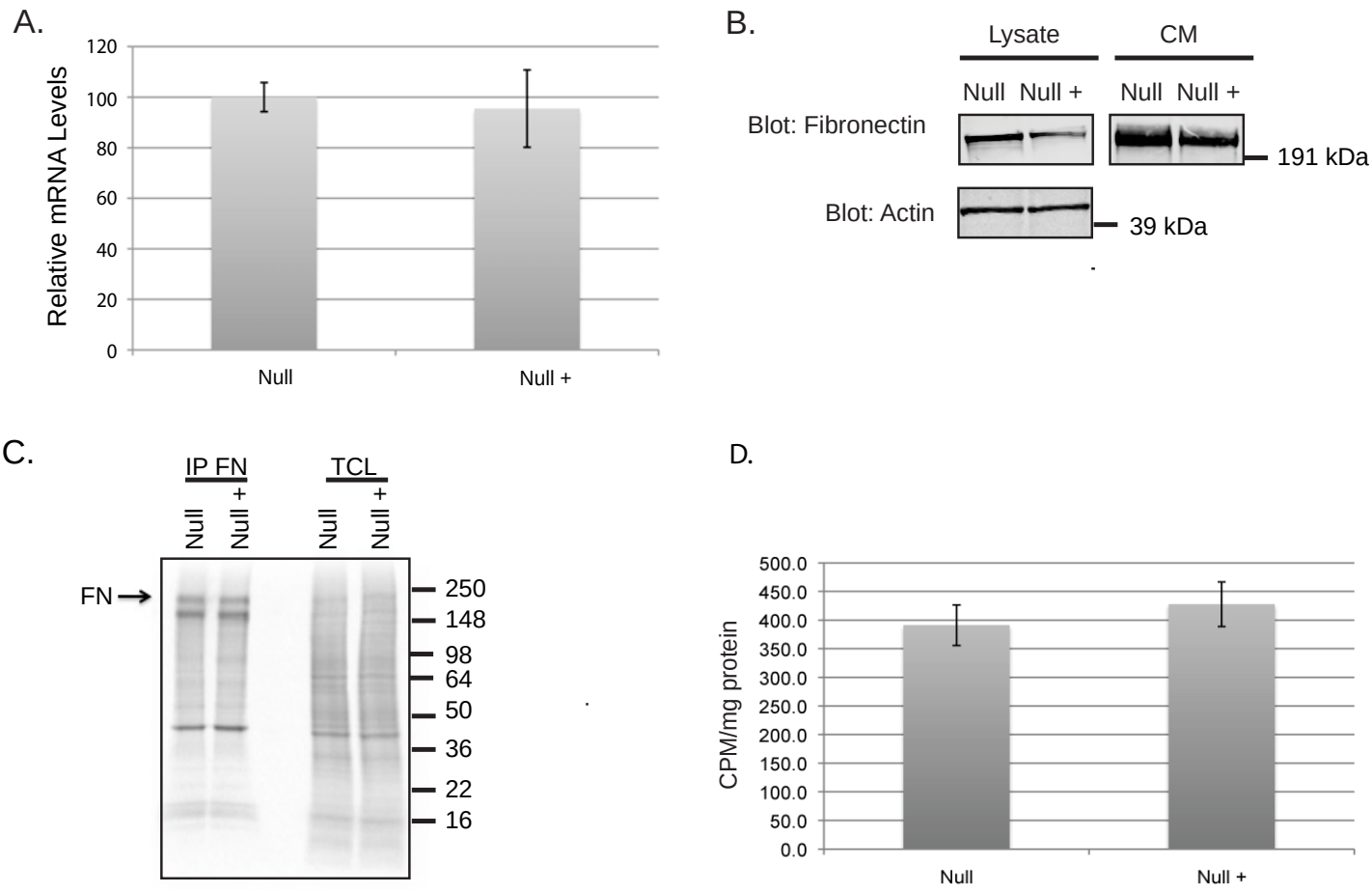
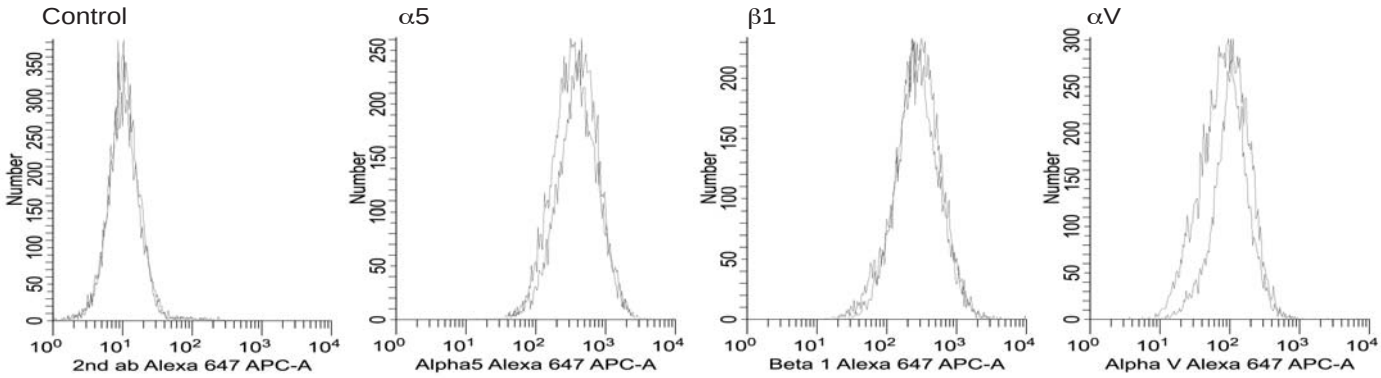

Control
Integrin β1
Integrin β3
Integrin β1 + β3
Null
Null +

Figure 5


A.
Relative mRNA Levels
120
100
80
60
40
20
0
Null
Null +
B.
Lysate
CM
Null
Null +
Null
Null +
Blot: Fibronectin
191 kDa
Blot: Actin
39 kDa
C.
IP FN
TCL
Null
Null +
Null
Null +
FN
250
148
98
64
50
36
22
16
D.
CPM/mg protein
500.0
450.0
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
Null
Null +

Supplemental Figure 1



Supplemental Figure 2