

SOFT 3D FIBRIN MATRICES DOWNREGULATE FAK EXPRESSION TO PROMOTE
SELF-RENEWAL OF TUMOR-REPOPULATING CELLS

BY

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THESIS

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Abstract

Tumor-repopulating cells are a highly tumorigenic subpopulation of cancer cells that exhibit stem cell-like properties (e.g. self-renewal). Previous reports have shown that soft 3D fibrin matrices promote self-renewal in TRCs by promoting histone 3 lysine residue 9 (H3K9) de-methylation and Sox2 expression [1]. However, the underlying mechanism(s) by which soft 3D fibrin matrices promote H3K9 de-methylation and Sox2 expression remain elusive. In this study we show that focal adhesion kinase (FAK) regulates Sox2 expression and H3K9 de-methylation through cell division control protein 42 homolog (Cdc42).

In comparison to control melanoma cells, TRCs exhibit low FAK and Cdc42 expression. Overexpressing FAK or Cdc42 in TRCs cultured in soft 3D fibrin matrices promotes H3K9 methylation, decreases Sox expression, and suppresses colony growth. Knocking down FAK or Cdc42 expression in control melanoma cells promotes H3K9 de-methylation, increases Sox2 expression, and enhances colony growth in stiff 3D fibrin matrices. Overexpressing Cdc42 in FAK-knock down control melanoma cells inhibits H3K9 de-methylation.

For my Mom, Dad, Brother, and Hunter...

Acknowledgments

Professor Ning Wang, thank you for taking a chance on me and giving me the biggest opportunity I have ever had in my life. Being a member of your lab is a privilege and I really enjoy playing basketball with you. I can honestly say I will never forget some of the advice you have given me. Thank you.

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All of you have helped me to develop as a person and as a researcher. Thank you all for being mentors and friends. I would like to especially thank Dr. Jihye Seong for getting us started on focal adhesion kinase. Her initial guidance was crucial to this project.

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Terminology

TRCs - subpopulation of cancer cells selected by soft 3D fibrin matrices. These cells are allowed to proliferate in soft fibrin matrices for 5 days before use for analysis or further experiments.

control melanoma cells - Murine melanoma cell line B16-F1 cultured on 0.1% gelatin or 50 ug/mL collagen I coated plastic.

expression – mRNA gene expression

Chapter 1: Introduction

Tumor-initiating cells (TICs) are a highly tumorigenic subpopulation of cancer cells that exhibit stem cell-like properties (e.g. self-renewal – cell division with maintenance of the undifferentiated state). These cells play a crucial role in macroscopic metastasis and the relapse of cancer after therapy [2]. Recently, a mechanical method was developed for selecting TICs from primary cancer cells and cancer cell lines by culturing individual cancer cells in soft (90-Pa) 3D fibrin matrices. The cells that survive and proliferate in the soft 3D fibrin matrices are termed tumor-repopulating cells (TRCs) based on their high efficiency in repopulating local and metastatic tumors in wild-type syngeneic and non-syngeneic mice [3].

It has been reported that soft (90-Pa) 3D fibrin matrices promote self-renewal in TRCs by promoting Sox2 expression via histone 3 lysine residue 9 (H3K9) de-methylation. In contrast, stiffer (1050-Pa) 3D fibrin matrices promote lower Sox2 expression via higher H3K9 methylation. Importantly, experiments in live mice have shown that higher Sox2 expression in TRCs leads to more efficient tumor formation *in vivo* [1]. These findings suggest that mechanical stimuli, such as matrix softness, play an important role in promoting stem cell-like properties that are essential to maintaining the high tumorigenic properties of TRCs *in vivo*. However, the mechanism(s) by which soft 3D fibrin matrices promote Sox2 expression via H3K9 de-methylation remain unknown. In this study we show that focal adhesion kinase (FAK) regulates Sox2 expression and H3K9 de-methylation through cell division control protein 42 homolog (Cdc42). Soft 3D fibrin matrices promote self-renewal by down regulating FAK expression, which lowers Cdc42 expression to mediate H3K9 de-methylation and Sox2 expression.

Chapter 2: Research Methodology

When we began to study the mechanism(s) by which soft 3D fibrin matrices promote Sox2 expression and H3K9 de-methylation in TRCs, we based our approach off of one very important previous finding: H3K9 de-methylation and Sox2 expression are responsive to the rigidity of 3D fibrin matrices [1]. This led us to hypothesize that the mechanism(s) by which soft 3D fibrin matrices promote Sox2 expression and H3K9 de-methylation could be controlled by mechano-sensors (molecules that mediate signal transduction in response to mechanical stimuli).

With the initial guidance of Dr. Jihye Seong, a former member of Professor Wang's lab, we began our mechano-sensor investigation with focal adhesion kinase (FAK). FAK is a non-receptor tyrosine kinase that has kinase-dependent and kinase-independent functions in many important cellular functions, such as cell migration, survival, and regulation of gene expression [4]. It is very well-known that FAK is sensitive and responsive to mechanical cues from the environment, such as substrate rigidity, and translates these cues into intracellular biochemical information to regulate various cellular functions [5,6]. In addition to its mechano-sensitivity, FAK has been shown to have abnormal expression levels in several different types of cancer [4].

Chapter 3: Results

Association between FAK expression and H3K9 de-methylation

We analyzed the FAK gene expression of control melanoma cells cultured in soft and stiff 3D fibrin matrices (90-Pa and 1050-Pa, respectively) after 5 days to determine if FAK is responsive to the 3D fibrin matrix rigidity. As previously mentioned, Sox2 expression and H3K9 methylation levels are responsive to fibrin matrix rigidity. Therefore, we expected that a mechano-sensor promoting these intracellular events would also be responsive to matrix rigidity. As shown in Figure 1, FAK expression is higher in melanoma cells cultured in stiff 3D fibrin matrices than in melanoma cells cultured in soft 3D fibrin matrices.

Knowing that both FAK expression and H3K9 methylation levels are low in soft 3D fibrin matrices, we overexpressed FAK in TRCs and re-plated them in soft 3D fibrin matrices to see if H3K9 methylation levels would change. Using a FRET-based biosensor that measures relative H3K9 methylation levels in live cells, we found that increased FAK expression could induce higher H3K9 methylation (Figure 2). We then analyzed the FAK expression in TRCs and control melanoma cells to determine if there is a difference between the two, as there is for Sox2 expression and H3K9 methylation. We found that FAK expression is greater in control melanoma cells than in TRCs and FAK expression increases in TRCs cultured on rigid plastic for 7 days (Figure 3). Previous findings show that TRCs gradually lose Sox2 expression and increase H3K9 methylation with culture time on plastic [1].

To further confirm a causal relationship, we knocked down FAK in control melanoma cells and measured H3K9 methylation levels and Sox2 expression. Previous reports have shown

that control melanoma cells exhibit very low Sox2 expression and high H3K9 methylation levels [3,1]. Knocking down FAK decreased H3K9 methylation levels (Figure 4) and increased Sox2 expression (Figure 5). Expression of H3K9 methyltransferases, G9a and Suv39h1, also decreased when FAK was knocked down in control melanoma cells (Figure 5). Previous findings have shown that decreased levels of G9a and Suv39h1 can lead to lower H3K9 methylation levels and increased Sox2 expression in control melanoma cells [1].

FAK promotes self-renewal in TRCs through Cdc42

To expand our understanding of how FAK mediates Sox2 expression and H3K9 de-methylation, we investigated possible intermediate proteins that may be in the signaling pathway. It is well-known that FAK can regulate cytoskeleton assembly and disassembly through interactions with Rho-family GTPases [7]. Also, previous findings have shown that Cdc42 (Rho-family GTPase) expression is lower in TRCs in comparison to control melanoma cells [1]. With this knowledge we decided to investigate if Cdc42 plays a role in mediating Sox2 expression and H3K9 de-methylation.

To confirm that Cdc42 is downstream of FAK, we knocked down FAK in control melanoma cells and analyzed Cdc42 expression (Figure 6). We then analyzed the gene expression of control melanoma cells cultured in soft and stiff 3D fibrin matrices to determine if Cdc42 expression was responsive to matrix rigidity. We found that Cdc42 expression increases with increasing matrix rigidity, which is consistent with FAK expression in response to increasing matrix rigidity (Figure 7 and Figure 1).

Knowing that both Cdc42 expression and H3K9 methylation levels are low in soft 3D fibrin matrices, we overexpressed Cdc42 in TRCs and re-plated them in soft 3D fibrin matrices to see if H3K9 methylation levels would change. Overexpressing Cdc42 in TRCs increased H3K9 methylation levels (Figure 8), which is consistent with FAK overexpression in TRCs (Figure 2). To further confirm a causal relationship, we knocked down Cdc42 in control melanoma cells and measured H3K9 methylation levels. Consistent with our FAK data, Cdc42 knockdown decreases H3K9 methylation and increases Sox2 expression (Figure 9 and Figure 10). Expression of H3K9 methyltransferases, G9a and Suv39h1, also decreased when Cdc42 was knocked down in control melanoma cells (Figure 10).

To further confirm that Cdc42 is downstream of FAK in the signaling pathway by which FAK regulates H3K9 methylation, we knocked down FAK in control melanoma cells while overexpressing Cdc42. As shown in Figure 11, overexpressing Cdc42 can inhibit H3K9 de-methylation caused by FAK knock down.

Functional role of FAK and Cdc42 in promoting self-renewal in TRCs

As previously stated, soft fibrin matrices promote self-renewal in TRCs by regulating Sox2 expression via H3K9 de-methylation. The functional role of Sox2 in regulating self-renewal *in vitro* has been shown by decreased colony growth of Sox2-knocked down TRCs in soft fibrin matrices [1]. To demonstrate the functional role of FAK and Cdc42 in regulating self-renewal in TRCs, we examined the effects of FAK and Cdc42 on colony growth in soft 3D fibrin matrices. Overexpressing FAK or Cdc42 in TRCs suppressed colony growth (Figure 12). This inhibitory effect is likely due to the suppression of Sox2 by FAK and Cdc42 overexpression (Figure 13).

Knocking down FAK or Cdc42 in control melanoma cells, which have high FAK and Cdc42 expression in comparison to TRCs, enhanced colony growth in stiff fibrin matrices (Figure 14) and increased Sox2 expression (Figure 15).

Chapter 4: Conclusion

In this study we show that FAK expression plays a crucial role in promoting self-renewal properties in TRCs cultured in soft 3D fibrin matrices. Low FAK or Cdc42 expression in TRCs or control melanoma cells promotes H3K9 de-methylation and Sox2 expression. Overexpressing FAK or Cdc42 in TRCs suppresses colony growth in soft 3D fibrin matrices and knocking down FAK or Cdc42 expression enhances colony growth of control melanoma cells cultured in stiff 3D fibrin matrices. Although unclear, it is likely that existing differentiated TRCs in the control melanoma cell population attribute to the enhanced colony growth of FAK-knock down or Cdc42-knock down control melanoma cells in stiff 3D fibrin matrices.

In contrast to our findings, it has been reported that FAK is overexpressed in many different types of cancers and contributes to tumor progression and metastasis [4]. This discrepancy between our findings may result from the fact that TRCs are only a small subpopulation of cancer cells in tumors. Previous studies show that TRCs cultured in soft 3D fibrin matrices, which have very low FAK expression, are much more efficient at forming metastatic tumors *in vivo* than control melanoma cells, which have relatively high FAK expression [3].

In the future we will need to conduct experiments in live mice to determine if overexpressing FAK in TRCs can inhibit local and metastatic tumor formation. It would also be interesting to study the effects of matrix dimensionality on FAK, Cdc42, H3K9 methylation, and Sox2. Previous studies have shown that control melanoma cells cultured in soft 3D fibrin

matrices are much more tumorigenic than genetically identical cells cultured on top of soft fibrin matrices [3].

Chapter 5: Materials and Methods

Cell culture

Murine melanoma cell line B16-F1 was purchased from American Type Culture Collection. Briefly, cells were cultured on rigid dishes with DMEM cell culture medium supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate and 0.1 mM penicillin/streptomycin at 37 °C with 5% CO₂. Cells were passaged every 3–4 days using TrypLE (Invitrogen). Glass or plastic culture dishes were coated with collagen-1 (50 µg/ml for glass or plastic dishes; 200 µg/ml for PA gels) or 0.1% Gelatin.

3D fibrin gel preparation

Salmon fibrinogen and thrombin were purchased from Reagent Proteins (CA, USA). Three-dimensional fibrin gels were prepared as described previously (Liu et al., 2012; Tan et al., 2014). In brief, fibrinogen was diluted into 2 mg/ml or 16 mg/ml with T7 buffer (pH 7.4, 50 mM Tris, 150 mM NaCl). Cells were detached from 2D rigid dishes. Fibrinogen and single cell solution mixture was made by mixing the same volume of fibrinogen solution and cell solution, resulting in 1 mg/ml or 8 mg/ml fibrin gels (the stiffness of 1 and 8 mg/ml fibrin gels is 90 Pa and 1050 Pa, respectively). 250 µl cell/fibrinogen mixture was seeded into each well of 24-well plate and mixed well with pre-added 5 µl thrombin (100 U/ml). The cell culture plate was then incubated in 37 °C cell culture incubator for 10 min. Finally, 1 ml of DMEM medium containing 10% fetal bovine serum and antibiotics was added.

Quantitative RT-PCR

Total mRNA was isolated from cells using the RNeasy Mini Kit (QIAGEN) according to the supplier's instruction. cDNA synthesis was conducted using the iScriptTM Reverse Transcription Supermix (Bio-Rad) according to the manufacturer's protocol. Real-time RT-PCR was performed using the SsoAdvancedTM Universal Probes Supermix (Bio-Rad). The data was normalized against mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All the primers for real-time RT-PCR were obtained from Bio-Rad (PrimePCRTM SYBR® Green Assay, Cat #: 100-25636).

FRET imaging

The H3K9 biosensor used in this study is reported elsewhere [1]. The biosensors were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. A Leica inverted fluorescence microscope integrated with Dual-View MicroImager system (Optical Insights) was used to capture CFP and YFP (YPet) emission images. CFP/YFP Dual EX/EM (FRET) (OI-04-SEX2) has the following filter sets: CFP: excitation, S430/25, emission S470/30; YFP: excitation, S500/20, emission S535/30. The emission filter set uses a 515-nm dichroic mirror to split the two emission images. For FRET imaging, each CFP (1344 pixels×512 pixels) and each YFP image (1344 pixels×512 pixels) were simultaneously captured on the same screen by using a charge-coupled device camera (C4742-95-12ERG; Hamamatsu) and a 40×, 0.55 numerical aperture air-immersion objective. A customized Matlab (Mathworks) programme was used to analyze CFP and YFP images and to obtain YPet/CFP (H3K9 methylation) emission ratios.

RNA interference

Cells were transfected with FRET biosensor, siRNA, or complementary DNA using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Silencer Negative Control No. 1 siRNA (Invitrogen, AM4611) was used as a negative control in RNAi experiment. The construct sequence is 5'-AGUACUGCUUACGAUACGGtt-3' for negative control siRNA, 5'-CGAGUAUUAAGGUCUUUCtt-3' for FAK siRNA #1 (Invitrogen, 157446), 5'-GCCUUAACAAUGCGUCAGUtt-3' for FAK siRNA #2 (Invitrogen, 157447), 5'-CCGCUAAGUUAUCCACAGAtt-3' for Cdc42 siRNA #1 (Invitrogen, 161124), and 5'-GGGCAAGAGGAUUAUGACAtt-3' for Cdc42 siRNA #2 (Invitrogen, 66023).

Statistical analysis

Two-tailed Student's t-test was used to conduct all the statistics.

Figures

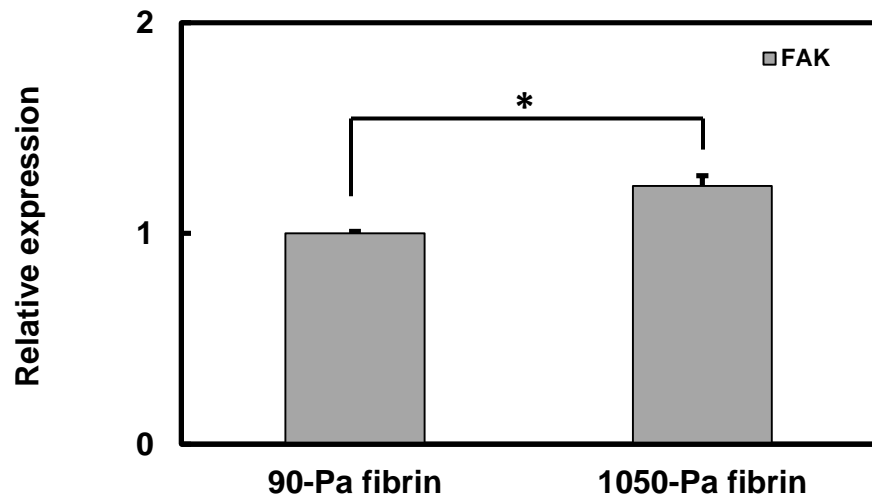


Figure 1: Melanoma cells have higher FAK expression in stiff fibrin gels. Control melanoma cells were cultured in 90-Pa and 1050-Pa 3D fibrin gels for 5 days. The mRNA was then extracted for analysis of the indicated gene by quantitative RT-PCR. Data was averaged from 3 independent experiments; * $p < 0.05$.

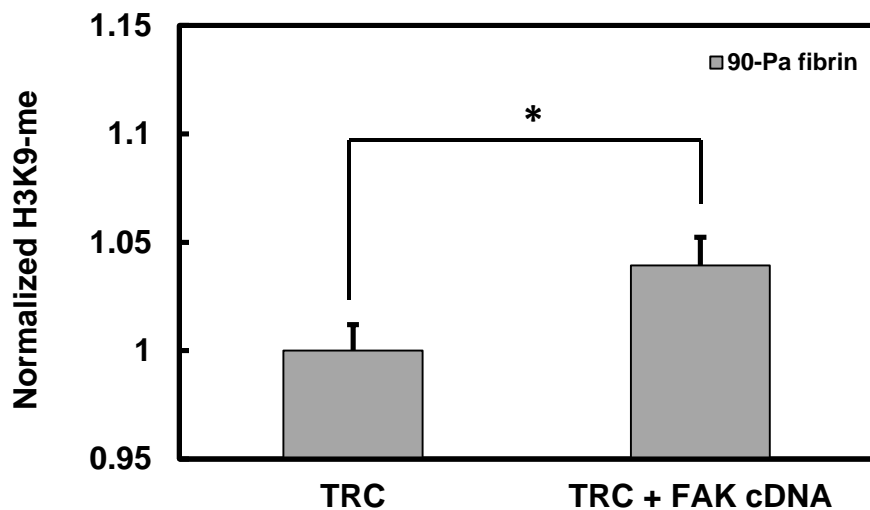


Figure 2: Overexpressing FAK in TRCs cultured in 90-Pa 3D fibrin matrices increases H3K9-methylation. H3K9 methylation was measured 15 hours after seeding TRCs in fibrin matrices using FRET-based biosensor; * $p < 0.05$.

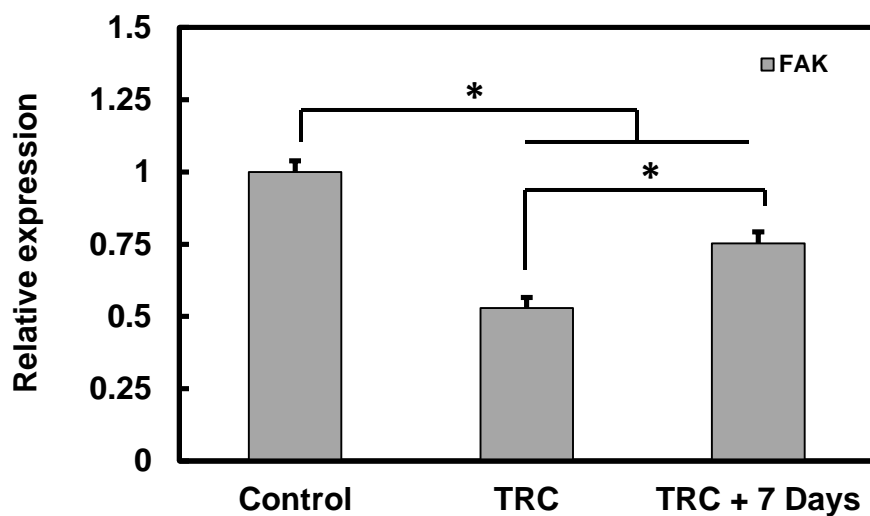


Figure 3: TRCs exhibit low FAK expression in comparison to control melanoma cells. Control B16-F1 melanoma cells were cultured in 3D soft (90-Pa) fibrin matrices (TRC) or on rigid dishes (Control). After 5 days, the mRNA was extracted for quantitative RT-PCR. TRCs cultured on plastic for 7 days exhibit increased FAK expression in comparison to TRCs in soft 3D fibrin matrices. Data averaged from 3 independent experiments; * $p < 0.05$.

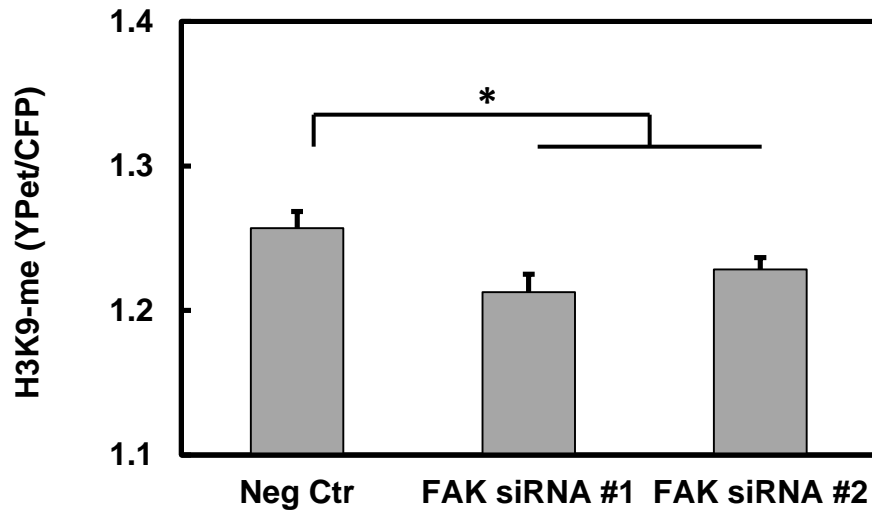


Figure 4: Knocking down FAK decreases H3K9 methylation in control melanoma cells cultured on 0.1% gelatin coated glass. H3K9 methylation was measured 15 hours after seeding cells on glass; * $p < 0.05$.

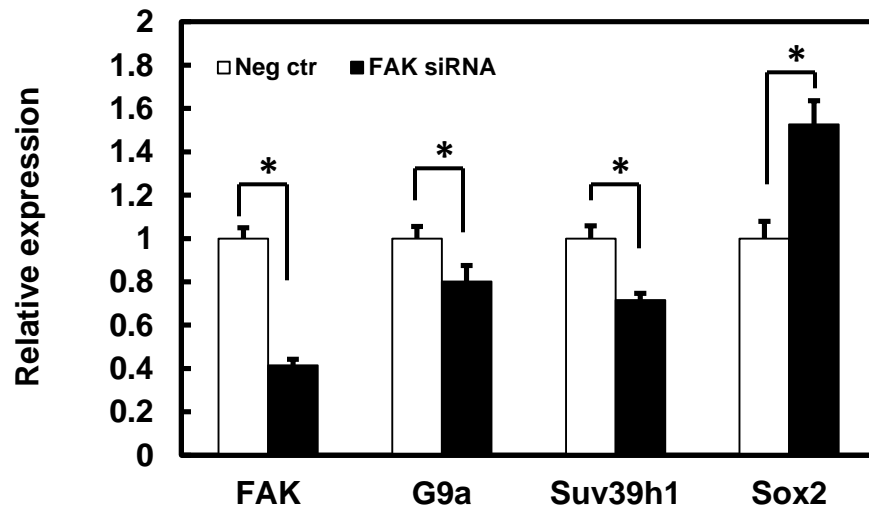


Figure 5: Knocking down FAK decreases expression of H3K9 methyltransferases, G9a and Suv39h1, and increases Sox2 expression in control melanoma cells cultured on 0.1% gelatin coated plastic; * $p < 0.05$.

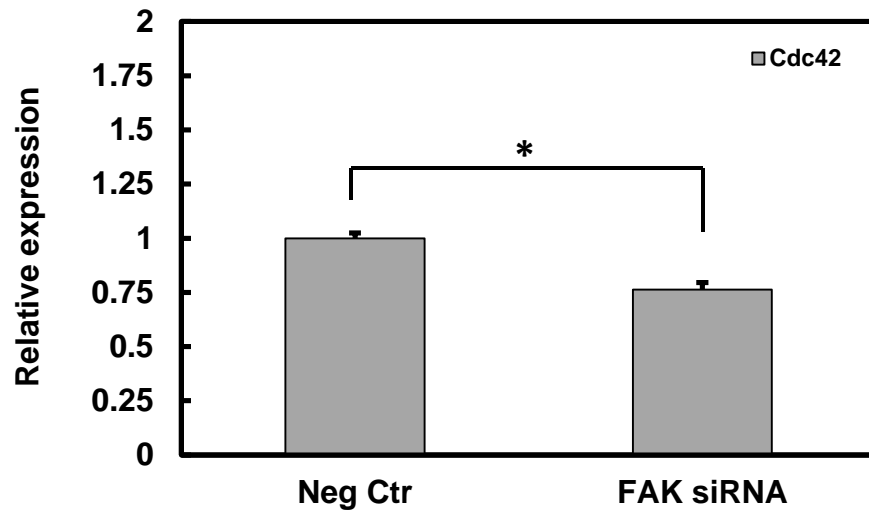


Figure 6: Knocking down FAK decreases Cdc42 expression in control melanoma cells cultured on 0.1% gelatin coated plastic; *p<0.05.

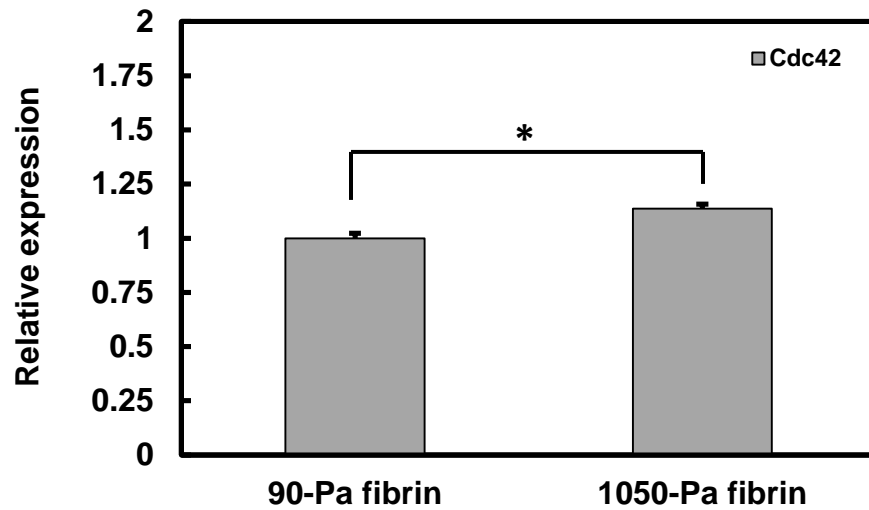


Figure 7: Melanoma cells have higher Cdc42 expression in stiff fibrin gels. Control melanoma cells were cultured in 90 Pa and 1050 Pa 3D fibrin gels for 5 days. The mRNA was then extracted for analysis of the indicated gene by quantitative RT-PCR. Data was averaged from 3 independent experiments; *p < 0.05.

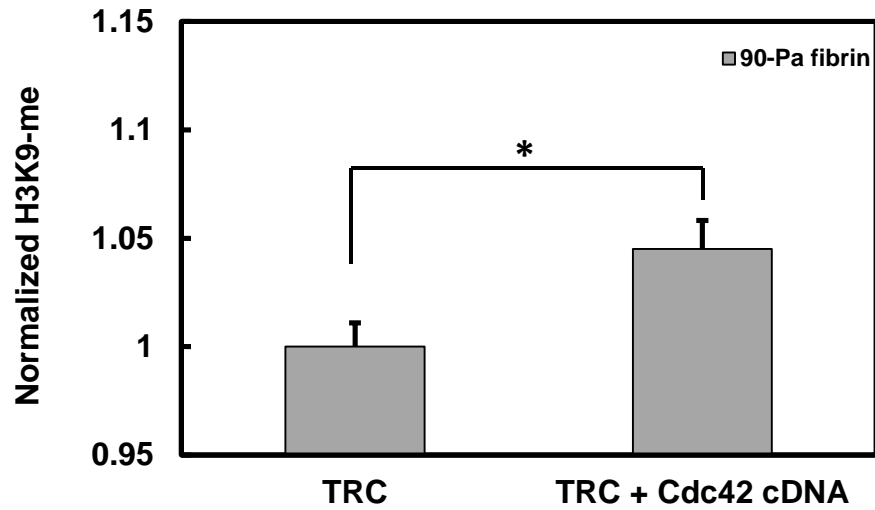


Figure 8: Overexpressing Cdc42 in TRCs cultured in 90 Pa 3D fibrin matrices increases H3K9-methylation. H3K9 methylation was measured 15 hours after seeding TRCs in fibrin matrices using FRET-based biosensor; * $p < 0.05$.

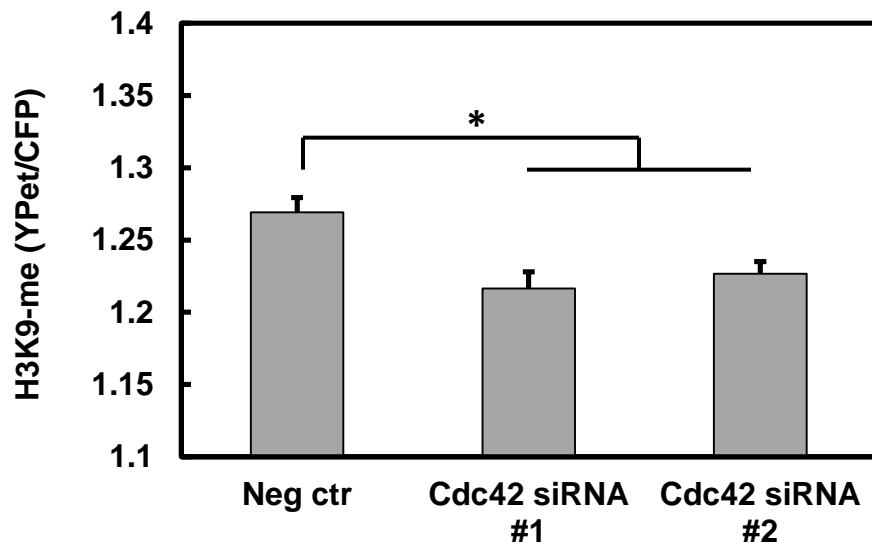


Figure 9: Knocking down Cdc42 decreased H3K9 methylation in control melanoma cells cultured on 0.1% gelatin coated glass. H3K9 methylation was measured 15 hours after seeding cells on glass; * $p < 0.05$.

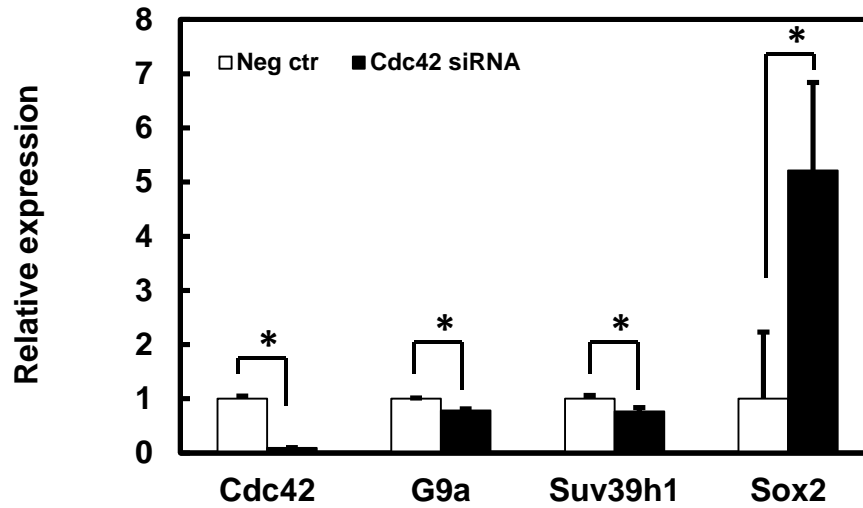


Figure 10: Knocking down Cdc42 decreased expression of H3K9 methyltransferases, G9a and Suv39h1, and increased Sox2 expression in control melanoma cells cultured on 0.1% gelatin coated plastic; *p<0.05.

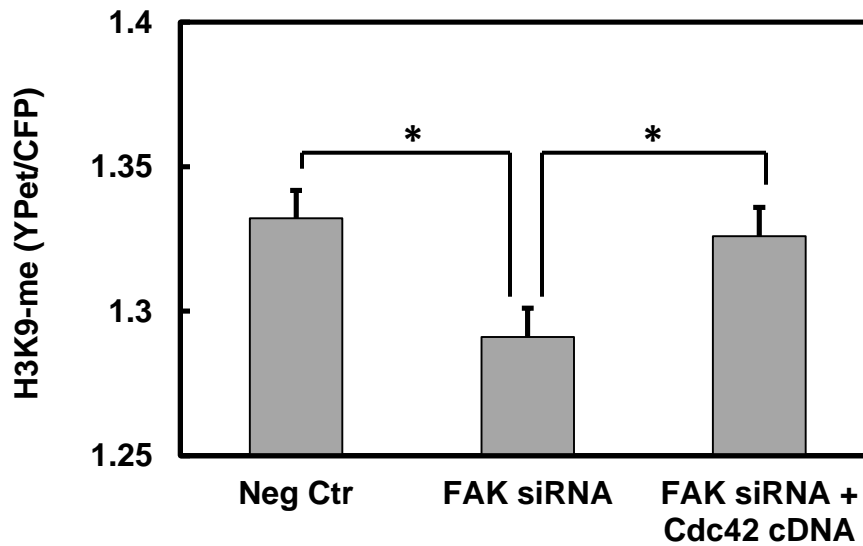


Figure 11: Overexpressing Cdc42 can inhibit H3K9 de-methylation caused by FAK knock down. H3K9 methylation was measured 15 hours after seeding cells on glass; *p<0.05.

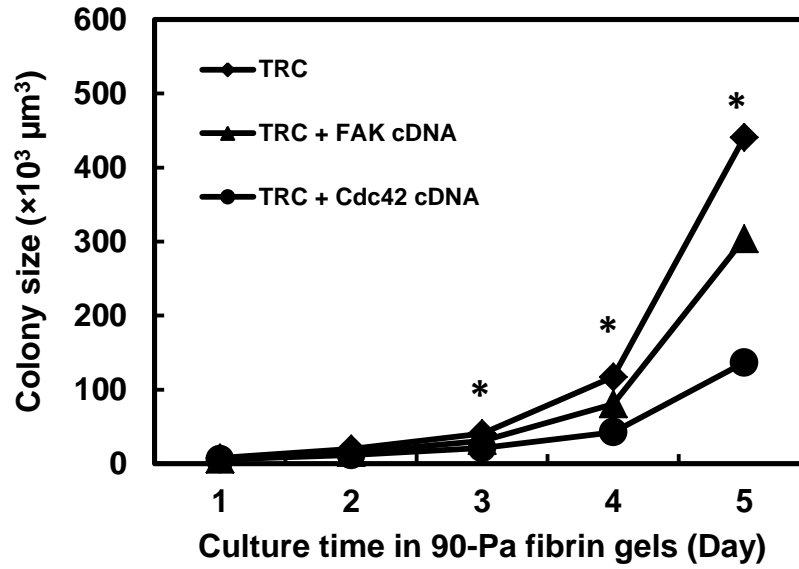


Figure 12: Overexpressing FAK or Cdc42 in TRCs suppresses colony growth in 90-Pa 3D fibrin matrices; * $p<0.05$.

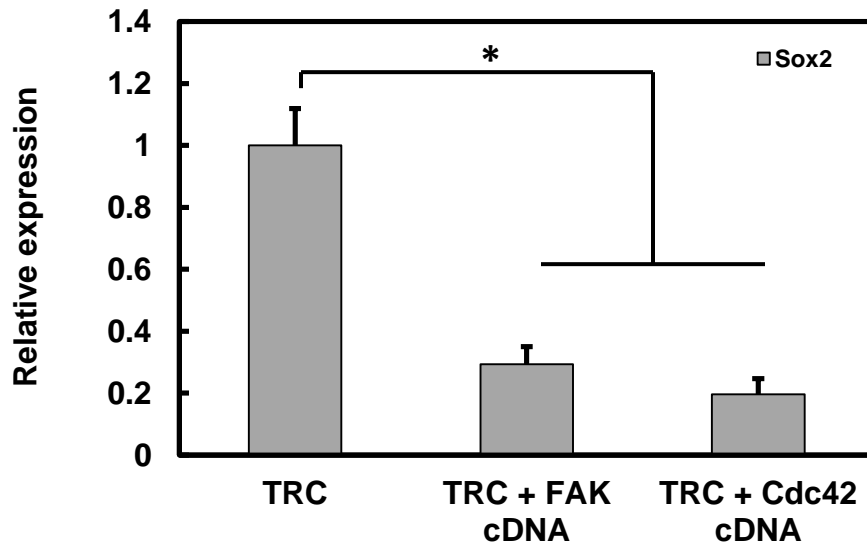


Figure 13: Overexpressing FAK or Cdc42 in TRCs decreases Sox2 expression in TRCs cultured for 5 days in 90-Pa 3D fibrin matrices; * $p<0.05$.

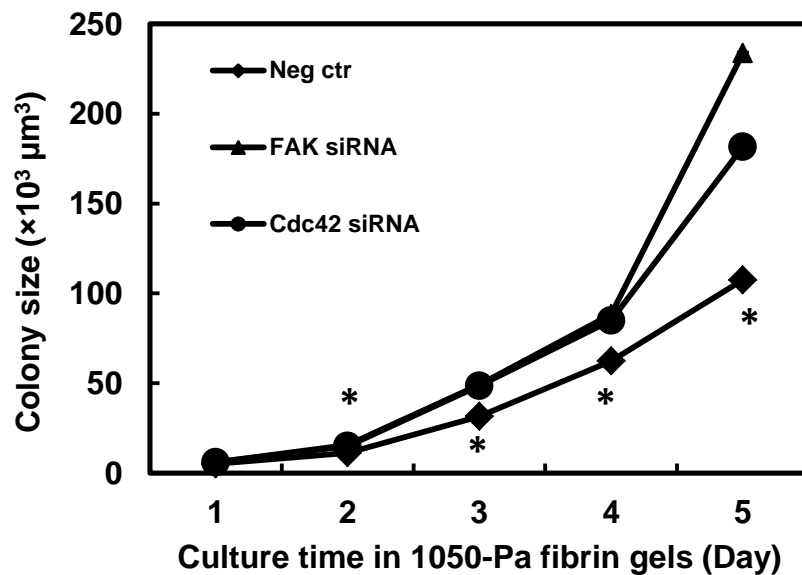


Figure 14: Knocking down FAK of Cdc42 in control melanoma cells enhances colony growth in 1050-Pa 3D fibrin matrices; * $p < 0.05$.

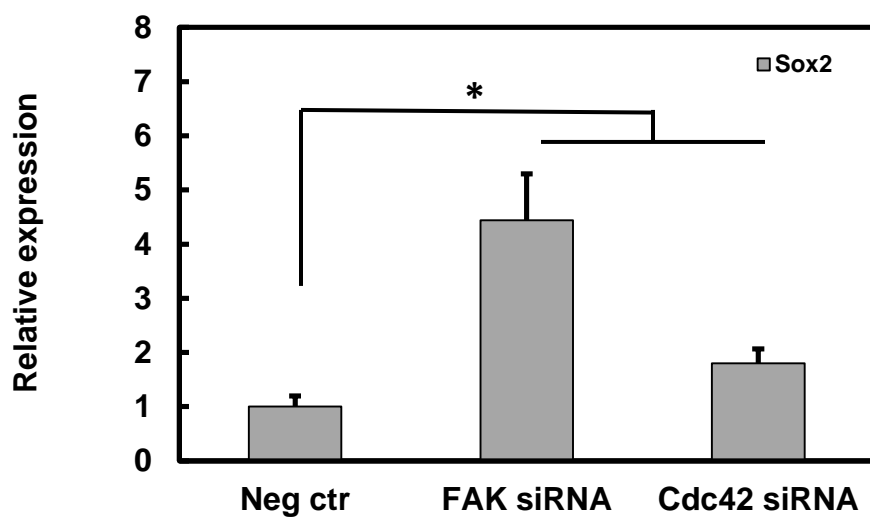


Figure 15: Knocking down FAK or Cdc42 increased Sox2 expression in control melanoma cells cultured in 1050-Pa 3D fibrin matrices for 5 days; * $p < 0.05$.

References

- [1] Tan, Y., Tajik, A., Chen, J., Jia, Q., Chowdhury, F., Wang, L., Chen, J., Zhang, S., Hong, Y., Yi, H., et al. (2014). Matrix softness regulates plasticity of tumour-repopulating cells via H3K9 demethylation and Sox2 expression. *Nat. Commun.* 5, 4619.
- [2] Zhou, B., Zhang, H., Damelin, M., Geles, K.G., Grindley, J.C., Dirks, P. (2009). Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. *Nat. Rev. Drug Discovery* 8, 806-823.
- [3] Liu, J., Tan, Y., Zhang, H., Zhang, Y., Xu, P., Chen, J., Poh, Y.-C., Tang, K., Wang, N., and Huang, B. (2012). Soft fibrin gels promote selection and growth of tumorigenic cells. *Nat. Mater.* 11, 734–741.
- [4] Sulzmaier, F., Jean, C., Schlaepfer, D. (2014) FAK in cancer: mechanistic findings and clinical applications. *Nat. Rev. Cancer* 14, 598-610.
- [5] Wang, H., Dembo, M., Hanks, S., Wang, Y. (2001) Focal adhesion kinase is involved in mechanosensing during fibroblast migration. *PNAS* 98 (20), 11295-11300.
- [6] Seong, J., Wang, N., Wang, Y. (2013) Mechanotransduction at focal adhesions: from physiology to cancer development. *J. of Cellular and Mol. Medicine* 17 (5), 597-604.
- [7] Mitra, S., Hanson, D., Schlaepfer, D. (2005) Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Bio.* 6, 56-68.