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Soft matrices downregulate FAK activity and H3K9 methylation to promote growth of tumor-repopulating cells

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Summary statement: Low FAK imparted by soft matrices mediates H3K9 demethylation and Sox2 expression through reduction of Cdc42 and RhoA to promote growth of tumor-repopulating cells.

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Abstract

Tumor-repopulating cells (TRCs) are a tumorigenic sub-population of cancer cells that drives tumorigenesis. We have recently reported that soft fibrin matrices maintain TRC growth by promoting histone 3 lysine 9 (H3K9) demethylation and *Sox2* expression and that *Cdc42* expression influences H3K9 methylation. However, the underlying mechanisms of how soft matrices induce H3K9 demethylation remain elusive. Here we find that TRCs exhibit lower focal adhesion kinase (FAK) and H3K9 methylation levels in soft fibrin matrices than control melanoma cells on 2D rigid substrates. Silencing FAK in control melanoma cells decreases H3K9 methylation, whereas overexpressing FAK in TRCs enhances H3K9 methylation. Overexpressing *Cdc42* or *RhoA* in the presence of FAK knockdown restores H3K9 methylation levels. Importantly, silencing FAK, *Cdc42*, or RhoA promotes *Sox2* expression and proliferation of control melanoma cells in stiff fibrin matrices, whereas overexpressing each gene suppresses Sox2 expression and reduces growth of TRCs in soft but not in stiff fibrin matrices. Our findings suggest that low FAK mediated by soft fibrin matrices downregulates H3K9 methylation through reduction of Cdc42 and RhoA and promotes growth of tumor-repopulating cells.

Introduction

Tumor-repopulating cells (TRCs) are a subpopulation of cancer cells within a tumor that possesses self-renewing capability and drives tumor progression and metastasis. It has been demonstrated that TICs (tumor-initiating cells) or TRC-like cells exist in several types of cancer, including blood (Lapidot et al., 1994), brain (Chen et al., 2012; Singh et al., 2004), skin (Driessens et al., 2012; Liu et al., 2012; Schatton et al., 2008), colon (Ricci-Vitiani et al., 2007), and intestine (Schepers et al., 2012). These tumorigenic cells are resistant to conventional chemotherapy and radiotherapy and speculated to be key players in cancer relapse (Baumann et al., 2008; Dean et al., 2005). Therefore, understanding the molecular mechanisms underlying TRC's high tumorigenicity is essential to achieve complete tumor eradication. We previously mechanically selected tumorigenic TRCs by culturing single cancer cells from cancer cell lines or primary tumors in three-dimensional (3D) soft fibrin matrices (Liu et al., 2012). A minor subpopulation of cancer cells survives soft fibrin matrices and one single cell grows into a round colony within 5 days. These selected spheroid cells exhibit high tumorigenic potential as they can efficiently repopulate tumors locally and in distant organs in wild-type syngeneic and non-syngeneic mice (Liu et al., 2012). Fibrin-selected TRCs express stem cell markers Sox2, Nestin, Bmi-1, and C-kit but no specific surface markers and exhibit much higher tumorigenicity than cancer cells isolated by conventionally used cell surface markers CD133 and CD44 (Liu et al., 2012). Soft fibrin matrices regulate TRC growth by promoting histone 3 lysine residue 9 (H3K9) demethylation that is associated with Cdc42 downregulation and Sox2 expression (Tan et al., 2014). However, the underlying mechanisms of how soft fibrin matrices regulate H3K9 demethylation remain elusive. Here we demonstrate that focal adhesion kinase (FAK)

regulates H3K9 methylation via Cdc42 and RhoA. Soft fibrin matrices maintain TRC growth by downregulating FAK, which in turn lowers Cdc42 and RhoA to mediate H3K9 demethylation and Sox2 upregulation.

Results

Soft matrices promote TRC growth via low FAK mediated Sox2 upregulation

We have reported that soft fibrin matrices decrease H3K9 methylation, increase Sox2 (a self-renewal gene) expression, and promote TRC's high tumorigenicity (Liu et al., 2012; Tan et al., 2014). However, the early cascade of matrix-cell mechanical signaling remains elusive. It is known that cells sense and respond to extracellular matrices mainly through integrin-mediated adhesion (Chen and Guan, 1994; Geiger et al., 2009; Seong et al., 2013; Wang et al., 2001). As one of the first proteins that interacts with cytoplasmic tails of integrins, FAK is abnormally expressed in several types of cancer and involved in tumor progression and metastasis (Itoh, 2004; McLean et al., 2005; Zhao and Guan, 2009). We hypothesized that FAK might play a critical role in regulating TRC growth. To demonstrate the functional roles of FAK and the downstream molecules Cdc42 and RhoA in TRC growth, we examined their effects on the colony growth in 3D fibrin matrices. Melanoma cells expressed higher FAK, Cdc42, RhoA, and methyltransferases and lower Sox2 in stiff than in soft 3D fibrin gels (Fig. S1A). These prompted us to explore whether overexpressing FAK, Cdc42, or RhoA in TRCs or silencing each gene affects colony growth in soft or stiff fibrin matrices. Overexpressing FAK or Cdc42 or transfecting a constitutively active construct RhoA V14 in TRCs suppressed the colony growth in soft but not in stiff fibrin matrices (Fig. 1A; Fig. S2A). This finding is supported by the results that overexpressing these genes inhibited TRC growth via suppression of Sox2 gene

expression in soft (Fig. 1B) but not in stiff fibrin gels (Fig. S2B), possibly because Sox2 is already very low in stiff fibrin gels (Tan et al., 2014). On the other hand, silencing FAK, Cdc42, or RhoA in control melanoma cells increased colony growth in stiff fibrin matrices (Fig. 1C), possibly due to upregulation of Sox2 expression (Fig. 1D). Interestingly, silencing FAK or Cdc42 but not RhoA promoted colony growth in soft fibrin matrices (Fig. S2C) without upregulating Sox2 gene expression (Fig. S2D), likely because Sox2 expression was already very high in the soft matrices (Tan et al., 2014) and thus could not be elevated further. These results also suggest that other Sox2-independent, growth-related gene(s) might contribute to melanoma cell growth in soft fibrin matrices. These findings suggest that FAK and its downstream molecules Cdc42 and RhoA may negatively regulate the growth of TRCs and that local matrix stiffness influences tumor growth by modulating Sox2 expression through these genes.

Association of low FAK activity with low H3K9 methylation in soft matrices

Next we examined whether matrix rigidity might influence FAK in melanoma cells. In comparison to control melanoma cells (melanoma cells that are cultured on 2D rigid plastic/glass), TRCs, melanoma cells that have been grown in soft fibrin matrices for 5 days, expressed lower FAK mRNA (Fig. 2A, top panel; Fig. S1B), lower level of FAK protein (Fig. S1D), and substantially lower Y-397 phosphorylation of FAK (Fig. 2A, bottom panel). Because TRCs could maintain their mechanical memory on rigid plastic/glass for 5 days (Tan et al., 2014), we re-plated TRCs back to 2D rigid glass for 7 days and found that FAK mRNAs were significantly increased (Fig. 2A). In addition, TRCs exhibited lower gene expressions of H3K9 methyltransferases EHMT2 and SUV39h1 (Fig. 2D, top panel; Fig. S1E) and lower H3K9 di- and tri-methylation (Fig. 2D, bottom panel).

To quantify FAK activity and H3K9 methylation at the single cell level, we used a FAK biosensor (Seong et al., 2011) and a H3K9 methylation biosensor (Tan et al., 2014), both of which are based on the principle of fluorescence resonance energy transfer (FRET). The FRET signal of FAK and H3K9 biosensors at the single cell level represents FAK Y-397 phosphorylation and H3K9 methylation, respectively, which has been well validated by western blotting assays (Seong et al., 2011; Tan et al., 2014). To better understand the underlying mechanisms, we set out to determine the dynamic changes of FAK activity and H3K9 methylation of melanoma cells at early time points (up to 48 hrs) after being cultured in soft fibrin gels. To rule out the effect of apoptosis, only cells with no detectable morphological signs of apoptosis (e.g., no shrinkage of cell volume and no blebbing) were chosen for experiments. Indeed, after FRET measurements, these same cells were able to continue to proliferate in the next few days. Our data show that both FAK activity and H3K9 methylation started to decrease as early as 3 hrs and stayed at the reduced levels up to 48 hrs after culture in soft (90-Pa) fibrin gels (Fig. 2B, E), which was further confirmed by western blotting assayed after 12 and 24 hrs in the soft gels (Fig. S1C, S1G). We recently has shown that TRCs lose Sox2 expression on rigid substrates (Tan et al., 2014). Here we found that FAK mRNA expression (Fig. 2A; Fig. S3A) and activity (Fig. 2A, C), gene expressions (Fig. 2D; Fig. S3D) and protein levels (Fig. S1F) of EHMT2 and SUV39h1, and H3K9 methylation levels (Fig. 2F) increased with culture time when TRCs were plated back (up to 7 days) onto the rigid glass. These results demonstrate that FAK activation levels and H3K9 methylation levels are closely associated in melanoma cells.

FAK regulates H3K9 methylation through Cdc42 and RhoA

Since FAK is a cytoplasmic protein tyrosine kinase that serves as a critical mechanosensor in integrin-mediated mechanotransduction (Geiger et al., 2009; Iskratsch et al., 2014; Wang et al., 2001), we wondered whether soft fibrin matrices induce H3K9 demethylation in the nucleus through FAK. Silencing FAK or expressing a mutant FAK, FRNK (Heidkamp, 2002), significantly decreased H3K9 methylation in control melanoma cells on the rigid glass (Fig. 3A; Fig. S4A, B). These data suggest that FAK activity regulates H3K9 methylation. Melanoma cells elevated their H3K9 methylation on 2D stiff substrates (8-kPa) or in 3D stiff fibrin gels (1050-Pa) compared to cells on soft substrates (0.15-kPa) or to cells in 3D soft (90-Pa) fibrin gels, which was abolished after FAK inhibition (Fig. 3B, C). In addition, TRCs increased their H3K9 methylation after being cultured on the rigid glass (Fig. 2F), which was completely abrogated by inhibiting FAK pharmacologically with 5 μM PF573228 (Fig. 3D). On the other hand, overexpressing FAK in TRCs enhanced H3K9 methylation both in 3D soft fibrin gels and on the rigid glass (Fig. 3E), whereas overexpressing FAK in melanoma cells increased H3K9 methylation in 3D soft fibrin gels but not on the rigid glass (Fig. 3F), likely due to the fact that FAK levels are already high in melanoma cells on the rigid glass. These results strongly suggest that FAK regulates H3K9 methylation.

As the downstream proteins of FAK and key cytoskeleton regulators (Han et al., 2011; McLean et al., 2005; Myers et al., 2012; Nobes and Hall, 1995), Cdc42 and RhoA gene expression levels were downregulated in TRCs compared to control melanoma cells and both were increased after plating TRCs back to the rigid glass (Fig. S3B, C). We have shown previously that silencing Cdc42 significantly decreased H3K9 methylation of control melanoma cells (Tan et al., 2014), which is supported by the data at the single-cell

level on the rigid glass (Fig. 4A; Fig. S4C). Here we further show that silencing Cdc42 abolished the elevation of H3K9 methylation on 2D stiff substrates (Fig. 4B) and in 3D stiff fibrin gels (Fig. 4C). Inhibition of Cdc42 pharmacologically with 10 μM ML-141 partially suppressed the elevation of H3K9 methylation when TRCs were cultured on the rigid glass (Fig. 4D). Furthermore, overexpressing Cdc42 significantly increased H3K9 methylation of TRCs on the rigid glass and in 3D soft fibrin gels (Fig. 4E). On the other hand, knocking down RhoA or inhibiting Rho kinase (ROCK) with 10 μM Y-27632 in control melanoma cells significantly decreased H3K9 methylation (Fig. 5A), while transfecting RhoA V14 in TRCs increased H3K9 methylation (Fig. 6C). These data suggest that both Cdc42 and RhoA regulate H3K9 methylation. To determine if FAK regulates H3K9 methylation through Cdc42 and RhoA, we knocked down FAK but overexpressed Cdc42 or transfected RhoA V14 in control melanoma cells and then cultured them on 2D substrates or in 3D fibrin gels. Overexpressing Cdc42 or transfecting RhoA-V14 increased H3K9 methylation on soft substrates (Fig. 4G, H; Fig. 5C, D) and restored H3K9 methylation levels even when FAK was silenced (Fig. 4F-H; Fig. 5B-D). Interestingly, when FAK was silenced, overexpressing Cdc42 had no effect in H3K9 methylation for cells on 2D soft substrates or in 3D soft fibrin gels (Fig.4G, H), whereas transfecting RhoA V14 significantly increased H3K9 methylation (Fig. 5C, D). These results indicate that RhoA but not Cdc42 may override the inhibitory effect of FAK knockdown in H3K9 methylation for cells on 2D or in 3D soft substrates. We further examined what would happen when FAK was silenced after we inhibited ROCK while Cdc42 was overexpressed or after we inhibited Cdc42 while RhoA V14 was expressed. Inhibiting ROCK or Cdc42 completely abolished the effect of Cdc42 overexpression or RhoA V14 transfection and

decreased H3K9 methylation (Fig. 4F; Fig. 5B), suggesting that there might be an interplay between Cdc42 and RhoA in regulating H3K9 methylation. This conclusion was further supported by the findings that silencing Cdc42 decreased RhoA expression (Fig. 7B) and silencing RhoA decreased Cdc42 expression (Fig. 7C). Transfecting RhoA V14 when Cdc42 was silenced or expressing Cdc42 cDNA when RhoA was silenced restored H3K9 methylation levels (Fig. S4E; Fig. 6B). These results suggest that Cdc42 and RhoA interact with each other to regulate H3K9 methylation.

Low FAK mediates H3K9 demethylation and Sox2 upregulation

We have recently reported that H3K9 demethylation increases Sox2 expression and growth of TRCs (Tan et al., 2014). We further asked whether and how low levels of FAK promote Sox2 expression in TRCs. In control melanoma cells, knocking down FAK inhibited the gene expressions of Cdc42, RhoA, EHMT2, and SUV39h1 and increased Sox2 gene expression (Fig. 7A); silencing Cdc42 decreased the gene expressions of FAK, RhoA, EHMT2, and SUV39h1 and increased Sox2 gene expression (Fig. 7B); inhibiting RhoA had no effect on FAK expression but significantly inhibited the gene expressions of Cdc42, EHMT2, and SUV39h1 and increased Sox2 expression (Fig. 7C). Our previous chromatin immunoprecipitation (ChIP) data showed that EHMT2 and SUV39h1 methylated H3K9 at the Sox2 promoter site blocks Sox2 expression and that silencing EHMT2 or SUV39h1 significantly increased Sox2 expression (Tan et al., 2014). These findings suggest that low FAK induces H3K9 demethylation at the Sox2 promoter region and thus Sox2 upregulation via downregulation of Cdc42 and RhoA.

Discussion

In the current study, we have found that compared to control melanoma cells on the rigid glass, TRCs exhibit low amount of total FAK and low Y397 phosphorylation of FAK and high Sox2 expression. Melanoma cells express higher FAK and lower Sox2 in stiff than in soft 3D fibrin matrices. Recently we have demonstrated that control melanoma cells on the rigid glass or plastic are differentiated cells as they express high levels of Mitf (a known master regulator of melanoma differentiation (Hemesath et al., 1994)) and low levels of Sox2 and TRCs are undifferentiated cells as they express low levels of Mitf and high levels of Sox2 (Tan et al., 2014). Importantly, we find in the current study that silencing FAK in control melanoma cells increases Sox2 expression in stiff but not in soft fibrin matrices and promotes growth in both soft and stiff fibrin matrices, whereas overexpressing FAK in TRCs decreases Sox2 expression and suppresses growth in soft but not in stiff fibrin matrices. Our data suggest that the local rigidity of 3D matrices is critical in regulating FAK activity to impact the expressions of Sox2 and/or other genes and TRC growth. In contrast, it has been reported that FAK is overexpressed in several types of cancer and responsible for high tumorigenicity and high invasive and metastatic potential of tumor cells (Ashton et al., 2010; Itoh, 2004; Lahlou et al., 2007; McLean et al., 2004; McLean et al., 2005; Pylayeva et al., 2009; Sulzmaier et al., 2014; Zhao and Guan, 2009). Furthermore, high FAK activity has been shown to promote growth of cancer stem cells (CSCs) that are usually selected by using cell surface markers (Fan et al., 2013; Luo et al., 2013; Schober and Fuchs, 2011; Thakur et al., 2015; Williams et al., 2015). These findings have led to the development of FAK inhibitors as a potential anticancer therapy (Cabrita et al., 2011; McLean et al., 2005; Sulzmaier et al., 2014). The discrepancy between previous reports and our current study may result from the fact that most of the total population of tumor

cells are probably differentiated tumor cells in those previous reports whereas only undifferentiated TRCs are analyzed in our present study. This explanation is supported by our findings that TRCs become differentiated after 7-day culture on the rigid glass (Tan et al., 2014) and significantly enhance their FAK activity (Fig. 2). Given the poor biological relevance of cell surface markers that are commonly used to select CSCs (Chaffer et al., 2011; Dieter et al., 2011; Gupta et al., 2011; Quintana et al., 2008), many of these CSCs (usually >1000 cells) are required for efficient tumor repopulation, while only as few as ten TRCs are needed to efficiently generate a tumor locally or at secondary sites after implantation locally or injection in the tail vein into immune-competent syngeneic and even non-syngeneic mice in our previous study (Liu et al., 2012). In addition, CSCs are heterogeneous in terms of their distinct effects on tumor growth (Dieter et al., 2011; Tang, 2012). Therefore, the differences in tumorigenicity and heterogeneity between TRCs and CSCs may lead to the disparity in FAK activity. In line with our current findings, it has been reported that high expression of FAK may not be a prognostic predictor for cancer patients (Furuyama et al., 2006; Schmitz et al., 2005; Theocharis et al., 2003); reduced FAK expression in FAK-heterozygous mice in fact enhances angiogenesis and the growth of melanoma and lung carcinoma (Kostourou et al., 2013); haematopoietic FAK deficiency promotes tumor metastasis (Batista et al., 2014); inhibition of FAK facilitates Ras-induced metastasis (Zheng et al., 2009); weak expression of FAK is also strongly associated with poor patient outcome (Gabriel et al., 2006). Interestingly, FAK expression is reduced in liver metastases compared with the matched primary tumors (Ayaki et al., 2001); inhibiting FAK cannot stop the progression to adenocarcinoma while FAK expression is required for the formation of neuroendocrine carcinoma (Slack-Davis et al., 2009). All these suggest

the differential roles of FAK in tumor progression. Importantly, we have demonstrated that Y397 phosphorylation of FAK is lost in undifferentiated TRCs, which further facilitates Sox2 expression and colony growth. Our results are consistent with the previous findings that the reduction in FAK activity promotes growth of stem cells (Jeon et al., 2012; Wrighton et al., 2014) while the increase in FAK activity facilitates their differentiation (Hayashi et al., 2007; Hunt et al., 2012; Murray et al., 2013). Recent reports have identified the important roles of kinase-independent scaffolding functions of FAK in cancer (Cance et al., 2013; Fan et al., 2013; Lim et al., 2008; Luo et al., 2013). These kinase-independent functions of FAK might partially explain regulation of Cdc42 and RhoA by FAK knockdown experiments, but the detailed mechanisms need to be explored in the future. Taken together, these findings suggest that the potential outcome of cancer therapy by inhibiting FAK may be much more complex than previously appreciated: it can lead to either inhibit or promote tumor growth, depending on the local rigidity of 3D matrices and types of integrin subsets (Seong et al., 2013). These findings may explain why FAK inhibition may not block tumorigenic cell proliferation as the current strategy in clinical trials of the candidate drugs to inhibit FAK does not work (Kaiser, 2015). In contrast to the previous findings that inhibition of FAK suppresses cancer progression, our current study reveals that silencing FAK can promote the expressions of Sox2 and/or other FAK-dependent genes and upregulate growth of tumorigenic cells. Nevertheless, we acknowledge that our current findings are limited only to melanoma cells and whether it can be extended to other types of cancer or to *in vivo* condition should be interrogated rigorously.

We have previously reported that β1 integrin is important in H3K9 methylation (Tan et al., 2014). In this study, we further show that H3K9 methylation and methyltransferases EHMT2 and SUV39h1 are higher in stiff (1050-Pa) than in soft (90-Pa) fibrin matrices, in which αvβ3 integrin is ligated. These data suggest that matrix stiffness is important in regulating H3K9 methylation, which cannot be explained by the absence of β1 integrin ligation. Based on our current findings, we propose a working model for the signaling pathways of soft-fibrin induced TRC's growth (Fig. 8). Cells sense mechanical forces from soft fibrin matrices via integrin subset αvβ3, which is the receptor of fibrin/fibrinogen and expressed in these melanoma cells (Liu et al., 2012). FAK, one of the first molecules downstream of integrin, serves as a mechanosensor and mediates force transduction into the cells (Geiger et al., 2009; Iskratsch et al., 2014; Wang et al., 2001). Soft fibrin matrices downregulate FAK that lowers Cdc42 and RhoA. The reduced levels of Cdc42 and RhoA reorganize the cytoskeletal structure, disassemble the stress fibers, and decrease the cytoskeletal tension (Hall, 1998; Nobes and Hall, 1995; Sit and Manser, 2011). This is supported by our previous findings that TRCs are much softer than control melanoma cells and do not exhibit mechanical stiffening responses due to low Cdc42 expression (Tan et al., 2014). Since TRCs and control melanoma cells exhibit similar integrin expression patterns (Liu et al., 2012), the reduction of FAK activity in soft fibrin matrices may be mediated by low forces and unrevealed αvβ3-dependent signaling pathways, which is supported by the previous findings that 3D soft fibrin but not collagen matrices can promote self-renewal and tumorigenicity of cancer cells (Liu et al., 2012). In addition, it is still not clear how Cdc42 and RhoA regulate H3K9 methylation in the nucleus. YAP/TAZ are potential candidates for relaying signals to the nucleus (Dupont et al., 2011). Given the

importance of the LINC (Linker of Nucleoskeleton and Cytoskeleton) complex in transmitting forces from the cytoskeleton to the nucleus (Wang et al., 2009; Dahl and Kalinowski, 2011; Guilluy et al., 2014), it is likely that LINC couples the propagation of low forces into the nucleus. Low forces further induce H3K9 demethylation at the region of Sox2 promoter (Tan et al., 2014), which promotes Sox2 expression and TRC growth. In addition, it is well-documented that histone modification including H3K9 methylation regulates nuclear architecture and chromatin assembly and condensation (Azzaz et al., 2014; Nakayama et al., 2001; Peng and Karpen, 2006; Towbin et al., 2012). The rapid reduction in H3K9 methylation of cancer cells after being cultured in soft fibrin matrices may indicate the reorganization of nuclear structures, which further influences gene expressions and cellular functions. Nevertheless, the signaling pathways of matrix-softness-dependent tumorigenicity remain to be elucidated in the future.

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 in 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). For all FRET imaging experiments, cells were detached using EDTA (Invitrogen) and the same number of cells were plated both on the indicated 2D substrates and in 3D fibrin matrices (6000 cells per glass-bottomed dish (cell density: 4000 cells/cm^2) or in one well of fibrin gels). Glass or plastic culture dishes and polyacrylamide (PA) gels were coated with collagen-1 (50 μg/ml for glass or plastic dishes; 200 μg/ml for PA gels) unless otherwise specified.

Polyacrylamide gel preparation

Polyacrylamide gels were prepared following the protocol reported previously (Wang and Pelham, 1998). The gel rigidity was varied by altering the concentrations of bis-acrylamide crosslinker (0.04%, 0.3%) and acrylamide (3%, 5%) and the corresponding gel rigidity is 0.15 kPa and 8.0 kPa, respectively.

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 mMTris, 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.

Reverse transcription-PCR and real-time RT-PCR analysis

Total mRNA was isolated from cells using the RNeasy Mini Kit (QIAGEN) according to the supplier's instruction. Reverse transcription (RT) was conducted using the iScript™ Reverse Transcription Supermix (Bio-Rad) and RT–PCR was performed using a PCR kit (QIAGEN) according to the manufacturer's protocols. Real-time RT–PCR was performed using the SsoAdvanced™ Universal Probes Supermix (Bio-Rad). The data were normalized against mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All the primers for real-time RT-PCR were obtained from Bio-Rad (PrimePCR™ SYBR® Green Assay, Cat #: 100-25636). The sequences of all the primers for RT–PCR were listed in the Table S1.

Western blotting assay

To quantify the expressions of FAK, H3K9 di- and tri-methylation, cells were lysed with 200 ml Laemmli sample buffer (Beyotime). 20 μl of each sample was separated by 8-15% SDS-PAGE, blocked with 5% BSA overnight at 4 ºC and incubated with primary antibodies to FAK (Rabbit, 1:1000, Abcam, ab40794), FAK-Y397 (Rabbit, 1:1000, Abcam, ab81298), H3K9 di-methylation (Rabbit, 1:300, Millipore, 17-648) and tri-methylation (Rabbit, 1:300, Millipore, 17-625) and GAPDH (Mouse, 1:1000, Abcam, ab8245) for 2 h

at room temperature. Primary antibodies were detected with goat anti-Rabbit IgG-HRP (1:2000, Santa Cruz, sc-2004) or anti-Mouse IgG-HRP (1:2000, Santa Cruz, sc-2005). The blots were developed using SuperSignal West Pico chemiluminescent substrate (Millipore).

FRET imaging

The Lyn-FAK and H3K9 biosensors used in this study were reported elsewhere (Seong et al., 2011; Tan et al., 2014). The biosensors were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. All the transfections in both control melanoma cells and TRCs were conducted on 2D rigid plastic/glass. No difference was observed in the transfection efficiency between control cells and TRCs, which was higher than 60%. Transfected cells were then plated on 2D substrates or in 3D fibrin matrices as indicated for FRET imaging where only transfected cells were measured. 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) program was used to analyze CFP and YFP images and to obtain YPet/CFP (for H3K9 methylation) or CFP/YFP (for FAK activity) emission ratios.

RNA interference

Cells were transfected with siRNAs and complementary DNA using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Silencer Negative Control No. 1 siRNA (Invitrogen, AM4611) was used a negative control in the RNAi experiment. The construct sequence is 5'-GGUGAUCCUUAUGCUGUUAtt-3' for EHMT2 siRNA (Invitrogen, 90322), 5'-GGUCCUUUGUCUAUAUCAAtt-3' for SUV39h1 (Invitrogen, 69566), and 5'-AGUACUGCUUACGAUACGGtt-3' for negative control siRNA. The construct sequence is 5'-CGAGUAUUAAAGGUCUUUCtt-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), 5'-GGGCAAGAGGAUUAUGACAtt-3' for Cdc42 siRNA #2 (Invitrogen, 66023), and 5'-GCCCUAUAUAUCAUUCUAGtt-3' for RhoA siRNA (Invitrogen, 165917).

Statistical analysis

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

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Competing interests

The authors declare no competing interests.

Author contributions

Y.T. and N.W. conceived the project; Y.T., A.R.W., Q.J., and N.W. designed the experiments; Y.T., A.R.W., and Q.J. carried out most of the experiments and analyzed the

data; J.W.C., J.J.C., and W.W.Z performed the western blotting assay and RT-PCR; Jian S. performed some experiments of real-time RT-PCR; J.S., A.T., and R.S. assisted and commented on the project; Y.T. and N.W. wrote the manuscript with inputs from other authors.

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Figure Legends

Fig. 1. FAK and Cdc42 and RhoA regulate growth of tumor-repopulating cells. Overexpression of FAK, Cdc42, or RhoA inhibits the colony growth (A) and Sox2 expression (B) of TRCs in soft fibrin matrices. TRCs were transfected with FAK cDNA, Cdc42 cDNA, or RhoA V14 and then plated into soft (90-Pa) fibrin matrices. Inhibition of FAK, Cdc42, or RhoA promotes the colony growth (C) and Sox2 expression (D) of control melanoma cells in stiff fibrin matrices. Control melanoma cells were transfected with negative control siRNA, FAK siRNA, Cdc42 siRNA, or RhoA siRNA and then cultured in stiff (1050-Pa) fibrin gels. In (A) and (C), colony growth was monitored from day 1 to day 5 (n=30 colonies per condition). In (A), significant differences between TRC and TRC+FAK cDNA, TRC+Cdc42 cDNA, or TRC+RhoA V14 from day 3 to day 5 (*$p<0.05$). In (C), significant differences between Neg Ctr and FAK siRNA #1, Cdc42 siRNA #1, or RhoA siRNA from day 2 to day 5 (*$p<0.05$). After 5 days, the mRNAs were extracted for analysis of Sox2 expression by real-time RT-PCR in (B) and (D) (n=3 independent experiments). *$p<0.05$.

Fig. 2. FAK activity and H3K9 methylation are tightly correlated. TRCs exhibit low FAK gene expression (A, top panel) and activity (A, bottom panel) and low gene expressions of H3K9 methyltransferases EHMT2 and SUV39h1 (D, top panel) and H3K9 methylation (D, bottom panel) in soft fibrin matrices. Control melanoma cells were cultured in 3D soft (90-Pa) fibrin matrices (TRC) or on rigid dishes (Control) for 5 days. TRCs were re-plated back on rigid dishes for additional 7 days to determine the rigid-dish effect on TRCs (TRC+7 days). The proteins and mRNAs of these cells were extracted for quantitative analysis of FAK and H3K9 methylation by western blotting assay and real-

time RT-PCR, respectively. n=3 independent experiments; *p<0.05. Control melanoma cells decrease FAK activity (B) and H3K9 methylation (E) after culture in soft fibrin matrices. Control cells were transfected with FAK or H3K9 biosensors on rigid dishes and then cultured in soft fibrin matrices. FRET images were taken at the indicated time points to quantify the FAK activity or H3K9 methylation. FAK activity and H3K9 methylation of control melanoma cells on glass coated with fibrinogen were used as a control (glass). TRCs increase FAK activity (C) and H3K9 methylation (F) after culture on rigid glasses coated with collagen-1. TRCs were re-plated back on to rigid glasses for 0, 1, 3, 5, and 7 days, respectively, when FAK activity and H3K9 methylation were measured with FRET biosensors. Representative FRET images were shown in the bottom panels of (B), (C), (E), and (F). n=35 cells per condition; *p<0.05. Scale bars: 10 μm in (B) and (C), FRET images of the cytoplasm; 5 μm in (E) and (F), FRET images of the nuclei.

Fig. 3. FAK activity regulates H3K9 methylation. (A) Silencing FAK decreases H3K9 methylation. Representative FRET images were shown in the bottom panel. Scale bars=5 μm. (B, C) FAK knockdown decreases H3K9 methylation on 2D stiff substrates (B) or in 3D stiff fibrin matrices (C) but not on 2D soft substrates or in 3D soft fibrin matrices. Control melanoma cells were transfected with H3K9 biosensors and negative control siRNA (Neg Ctr), or FAK siRNA #1 or #2, or a negative mutant FRNK. Cells were then plated on rigid glass (A), soft (0.15 kPa) and stiff (8 kPa) polyacrylamide gels (B), or in 3D soft (90-Pa) and stiff (1050-Pa) fibrin matrices (C). All the FRET imaging experiments were conducted 15 hrs after cell seeding throughout this paper unless otherwise specified. (D) Inhibition of FAK suppresses the increase of H3K9 methylation of TRCs on the rigid glass. TRCs were cultured on rigid glass for 7 days with (TRC+7 days + FAK inhibitor) or

without (TRC+7 days) 5 μM FAK inhibitor PF573228. H3K9 methylation of TRCs, TRC+7 days, and TRC+7 days + FAK inhibitor was measured with H3K9 biosensors. (E, F) Overexpression of FAK increases H3K9 methylation. TRCs (E) or control melanoma cells (F) were transfected with FAK cDNA and H3K9 biosensors and re-plated on the rigid glass or in soft fibrin matrices. In each sub-figure, n=35 cells per condition; *$p<0.05$.

Fig. 4. FAK regulates H3K9 methylation through Cdc42. (A) Silencing Cdc42 decreases H3K9 methylation. Representative FRET images were shown in the bottom panel. Scale bars=5 μm. Knockdown of Cdc42 in control melanoma cells inhibits the increase of H3K9 methylation on 2D stiff substrates (B) and in 3D stiff fibrin matrices (C). Control melanoma cells were transfected with H3K9 biosensors and negative control siRNA or Cdc42 siRNA. These cells were then plated on glass (A) or polyacrylamide gels (B) or in fibrin matrices (C). FRET imaging was conducted to quantify H3K9 methylation. (D) Inhibition of Cdc42 suppresses the increase of H3K9 methylation of TRCs on the rigid glass. TRCs were cultured on the rigid glass with or without 10 μM Cdc42 inhibitor ML-141. H3K9 methylation was measured after 7 days. (E) Overexpression of Cdc42 increases H3K9 methylation of TRCs. TRCs were transfected with Cdc42 cDNA and H3K9 biosensors and then plated on glass or in 90-Pa soft fibrin gels. (F-H) Overexpression of Cdc42 restores H3K9 methylation levels inhibited by FAK knockdown. Control melanoma cells were transfected with H3K9 biosensors and negative control siRNA, or FAK siRNA #1, or Cdc42 cDNA, or FAK siRNA #1 plus Cdc42 cDNA. Cells were then plated on the rigid glass (F), polyacrylamide gels (G), or in 3D fibrin matrices (H). In (F), control cells transfected with FAK siRNA and Cdc42 cDNA were treated with 10 μM Rho kinase

(ROCK) inhibitor Y-27632 for 1 hr before H3K9 methylation was measured. In each sub-figure, n=35 cells; *p<0.05.

Fig. 5. FAK regulates H3K9 methylation through RhoA. (A) Silencing RhoA decreases H3K9 methylation. Control melanoma cells were transfected with H3K9 biosensors and negative control siRNA or RhoA siRNA and then re-plated on glass. 10 μM ROCK inhibitor Y-27632 was used to treat the cells for 1 hr before H3K9 methylation was measured. Representative FRET images were shown in the bottom panel. Scale bars=5 μm. (B-D) Overexpression of RhoA restores H3K9 methylation levels inhibited by FAK knockdown. Control melanoma cells were transfected with H3K9 biosensors and negative control siRNA, or FAK siRNA #1, or RhoA-V14, or FAK siRNA and RhoA-V14. Cells were then plated on the rigid glass (B), polyacrylamide gels (C), or in 3D fibrin matrices (D). In (B), control cells transfected with FAK siRNA and RhoA-V14 plasmids were treated with 10 μM Cdc42 inhibitor ML-141 for 1 hr before H3K9 methylation was measured. In each sub-figure, n=35 cells; *p<0.05.

Fig. 6. RhoA regulates H3K9 methylation through Cdc42. (A) Knockdown of RhoA efficiently decreases RhoA expression. n=3 independent experiments. (B) Overexpression of Cdc42 restores H3K9 methylation inhibited by RhoA knockdown. Control melanoma cells were transfected with H3K9 biosensors and negative control siRNA, RhoA siRNA, or RhoA siRNA and Cdc42 cDNA. These cells were re-plated on rigid glass for 15hrs, when H3K9 methylation was measured. (C) Overexpression of RhoA V14 increases H3K9 methylation of TRCs. TRCs were transfected with H3K9 biosensors and RhoA V14 and then plated on glass. H3K9 methylation was measured 15 hrs after cell seeding. For each condition of (B) and (C), n=35 cells; *p<0.05.

Fig. 7. Sox2 expression is regulated by low FAK mediated H3K9 demethylation via Cdc42 and RhoA. (A) Knockdown of FAK decreases the expressions of Cdc42, RhoA, EHMT2, and SUV39h1 and increases Sox2 expression. (B) Knockdown of Cdc42 decreases the expressions of FAK, RhoA, EHMT2, and SUV39h1 and increases Sox2 expression. (C) Knockdown of RhoA decreases the expressions of Cdc42, EHMT2, and SUV39h1 and increases Sox2 expression. Control melanoma cells were transfected with negative control siRNA, or FAK siRNA, or Cdc42 siRNA, or RhoA siRNA. The mRNAs were then extracted for analysis of the indicated genes by real-time RT-PCR. In each sub-figure, n=3 independent experiments; *$p<0.05$.

Fig. 8. The proposed working model of soft-fibrin regulated TRC growth. Soft fibrin matrices mediate mechanical forces via $\alpha v\beta 3$ integrin and decrease FAK activity, Cdc42, and RhoA that may reorganize the cytoskeletal structure and lower the cellular tension. The low tension is likely transmitted through the LINC (Linker of Nucleoskeleton and Cytoskeleton) complex into the nucleus and then induces H3K9 demethylation at the region of Sox2 promoter, which enhances Sox2 expression and TRC growth.