TGF-β1-mediated activations of c-Src and Rac1 modulate levels of cyclins and p27Kip1 CDK inhibitor in hepatoma cells replated on fibronectin.

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ABSTRACT

Integrin-mediated cell adhesion transduces signals to regulate actin cytoskeleton and cell proliferation. While understanding how integrin signals cross-talk with the TGF-β1 pathways, we observed lamellipodia formation and cyclins regulation in Hep3B cells, following TGF-β1 treatment. To answer if integrin signaling via actin organization might regulate cell cycle progression after TGF-β1 treatment, we analyzed cross-talk between the two receptor-mediated pathways in hepatoma cells on specific ECMs. We found that basal and TGF-β1-mediated activation of c-Src and Rac1, expression of cyclins E and A, and suppression of p27Kip1 were significant in cells replated on fibronectin, but not in cells on collagen I, indicating a different integrin-mediated cellular response to TGF-β1 treatment. Levels of tyrosine phosphorylation and actin-enriched lamellipodia on fibronectin were also more prominent than in cells on collagen I. Studies using pharmacological inhibitors or transient transfections revealed that the preferential TGF-β1 effects in cells on fibronectin required c-Src family kinase activity. These observations suggest that a specific cross-talk between TGF-β1 and fibronectin-binding integrin signal pathways leads to the activation of c-Src/Rac1/actin-organization, leading to changes in cell cycle regulator levels in hepatoma cells. Therefore, this study represents another mechanism to regulate cell cycle regulators when integrin signaling is collaborative with TGF-β1 pathways.
INTRODUCTION

Cell proliferation is a tightly controlled and ordered process including multiple checkpoints responsible for the regulation of abnormal cell cycle progression. Transition between cell cycle phases is regulated by biochemically-coordinated actions of cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs), all of which can in turn be modulated by signal transduction initiated from extracellular growth factors [1]. So far, the G1 to S phase transition through the restriction (R) point has been shown to be regulated by mitogenic reagents, intact cytoskeletal network, as well as cell adhesion [2-6].

Cell adhesion is mediated by the engagement of cells to ECMs through integrins [5, 7]. Integrins are a heterodimeric cell adhesion receptor consisting of an α subunit and a β subunit. Approximately two dozen combinations from 18 α and 8 β subunits are known to assemble. These interactions are well-known to regulate diverse biological functions of cells including cell adhesion, spreading, proliferation, migration, survival, gene transcription, and cell cycle progression [5, 8, 9]. Integrin-mediated signaling include direct signaling transduced by integrins engaging with ECMs leading to downstream intracellular signaling molecules, and collaborative signaling where integrins co-signal with other receptor-mediated signaling pathways, such as receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs). Both integrin-mediated direct signaling and collaborative (indirect) signaling modulate cell adhesion and spreading, which are important events in the mitogenic response of cells to extracellular stimuli including growth factors [4, 5, 10, 11].

On the other hand, integrin-mediated cell adhesion is well-known to activate small (RhoA) GTPase family members, such as RhoA, Rac1, and CDC42, which regulate the actin cytoskeletal organization and lead to stress fiber/focal adhesions turnover, lamellipodia, and filopodia formation, respectively [12]. Interestingly, activated GTPases are shown to regulate cell cycle progression, by modulating either transcriptionally or translationally the
level of the G1 phase cyclin, cyclin D, or by inducing the timing of cyclin D expression, during G1 [4, 13-17]. It may be interesting to know how actin reorganization via activation of these GTPases may regulate expression of cell cycle regulators, and how such a regulatory relationship depends on signaling cross-talk between integrins and TGF-β1 pathways.

Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that inhibits epithelial cell growth and stimulates growth of fibroblasts as well, by binding to a heterodimeric receptor consisting of both type 1 (TβR1) and type 2 (TβRII) serine/threonine kinase receptors. On binding TGF-β1, the TβRII transphosphorylates and activates TβRI. Activation of the receptor complex propagates intracellular signal transduction involving SMAD proteins, which regulate numerous developmental and homeostatic processes by regulating gene expression [18-20]. Recently, TGF-β1-mediated but SMAD-independent signaling pathways have also been observed in various cell culture systems that involve numerous intracellular signaling molecules including Erk1/2, JNKs, and p38 MAPK, c-Src, Rho proteins, FAK, and Ras [21, 22]. Similar to RTKs or GPCRs, TGF-β1 receptor mediated signal pathway is also collaborative with integrin signaling. It was previously reported that treating mammary epithelial cells with TGF-β1 resulted in an epithelial-mesenchymal transdifferentiation (EMT), which required both a functional integrin β1 and active p38 MAPK [23].

We observed that Hep3B cells treated with TGF-β1 resulted in lamellipodia formation and increased cyclins D and E. Integrin-mediated cell adhesion signaling also appeared to affect lamellipodia formation through actin reorganization and cyclin levels. Therefore, we tried to answer how the integrin- and TGF-β1-mediated signal cross-talk lead to lamellipodia formation and increased expressions of cell cycle regulators. This study revealed that TGF-β1 induced lamellipodia formation through activation of c-Src and subsequently Rac1 in cells replated on fibronectin, but not on collagen I. The different TGF-β1 effects on fibronectin
correlated with increased expression of cyclins E and A and suppression of p27\textsuperscript{Kip1} CKI, in hepatoma cells. These indicate another putative cell cycle regulation mechanism in which cell cycle regulators are modulated through c-Src/Rac1/actin reorganization when TGF-β1 pathways encounter specific integrin signaling activity.
MATERIALS AND METHODS

Cells: Hep3B and Hur7 hepatocarcinoma cells were purchased from ATCC and grown in 37°C and 5% CO₂ in RPMI-60 culture media containing 10% fatal bovine serum.

Cell lysate preparation and Western Blot: After cells in normal cultures were treated without or with TGF-β1 for 48 hr, or after cells were replated and incubated on collagen I or fibronectin in the absence of serum for 15 hr, cell lysates were prepared as described in previous studies [24, 25]. Briefly, cells trypsinized were incubated in suspension within serum free media plus 1% BSA (replating media) for 1 hr before replating on indicated ECMs (15 µg/ml of fibronectin or collagen type 1, Chemicon, Temecula, CA). In certain cases, cell suspensions were pretreated with pharmacological inhibitors for 30 min before replating on ECMs. TGF-β1 (5 ng/ml, Chemicon) was added directly to the media at the same time of replating. Cell lysates were prepared 15 hr after the replating and treatment. Lysates were used in Western blots using anti-Erk1/2, phospho-Erk1/2, phospho-Y⁴¹⁶Src (Cell Signaling Technology, Beverly, MA), p38, phospho-p38, c-Src, p15⁴ᴷ, p16⁴ᴷ, Cyclin D1, Cyclin E, Cyclin B, Cyclin A (Santa Cruz Biotechnology, Santa Cruz, CA), PKB/Akt, phospho-S⁴⁷³PKB/Akt (New England Biolabs, Beverly, MA), Rac1, FAK, p21⁵³, p27⁵³, β-tubulin (BD Transduction Laboratories, San Diego, CA), or phospho-Y³⁹⁷FAK (Biosource International, Camarillo, CA). In some cases that nitrocellulose membrane was reprobed with another primary antibody, the membrane was stripped by incubation in a stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol) at 65°C for 30 min, washed for 1 hr (3 times x 20 min) with Tris-buffered saline containing 0.05% Tween-20 (TBST), reblocked with TBST containing 1% BSA plus 2% nonfat milk proteins, and then reprobed with another primary antibody.

Flow cytometry: Flow cytometric measurements of integrin subtypes on cells were performed as previously described [24]. For the studies of TGF-β1 effects on integrin expression levels,
Hep3B cells were treated with TGF-β1 (5 ng/ml) in a serum containing normal condition for 48 hr before harvesting for flow cytometric measurements. The raw data were analyzed by using WinMDI software program (version 2.7, Scripps Institute, San Diego, CA).

*Phase-contrast imaging:* Cells replated on the indicated substrates and treated with TGF-β1 were imaged by using a phase-contrast microscope.

*Rac1 in vitro pull down assay:* Rac1 activity in the cells under various conditions was examined via a pull-down assay by using an activation specific probe GST-PAK1 (76-150), as described previously [17]. Lysates from diverse treatment conditions were prepared as explained above.

*BrdU-incorporation Assay:* Cells were replated on either collagen I- or fibronectin-coated coverslips, and concomitant TGF-β1 treatment and BrdU addition were done by adding them directly to the replating media. After incubation at 37°C for 15 hr, cells were fixed with 100% cold methanol for 10 min at room temperature (RT). Fixed cells were washed twice with ice-cold PBS and then incubated with anti-BrdU solution containing nuclease (Roche, Indianapolis, IN) in a humid chamber for 30 min at 37°C. Cells were then stained with anti-mouse IgG conjugated with FITC (Jackson Laboratory, Bar Harbor, ME) diluted with PBS/1% BSA for 1 hr at room temperature. After incubation in the dark, cells were washed with PBS and mounted in Biomedia®. Using a Bio-Rad MRC-500 microscope, positive cells were visualized. At the same time, phase-contrast images of the same area were recorded. Five independent areas were imaged and used for calculation of mean values of BrdU-incorporated cells in each condition.

*Indirect immunofluorescence:* Cells replated onto specific ECM proteins were fixed in 3.7% formaldehyde in PBS for 10 min at RT. Cells were then permeabilized with 0.5% Triton X-100 in PBS for 5 min at RT. Then, cells were incubated for 1 hr at RT with anti-phosphotyrosine (1:500, BD Transduction Laboratories) or phalloidin-Rhodamine (1:200,
Sigma). After incubation, the cells on coverslips for phosphotyrosine were washed 3 times with PBS and then incubated with 1:200 FITC-conjugated anti-mouse IgG (Jackson Lab.) for 1 hr, respectively. The cells were then washed with PBS and nano-pure H₂O before mounting. Cell images were obtained using a Bio-Rad MRC-500 microscope.

**Construction of c-Src mutants:** The cDNA for chicken c-Src (kindly gifted by Dr. Jun-Lin Guan, Cornell University, Ithaca, NY) was used for point mutations of inactive Y416F or active Y527F c-Src via using of QuikChange™ Site-directed mutagenesis kit (Stratagene), according to the protocol by manufactures.

**Transient transfection:** Transient transfections with mutant forms of c-Src (inactive c-SrcY416F or active Y527F) were performed by using LipofectAMINE 2000 reagent, according to the manufacturer’s protocols (Invitrogen). After transfection of each construct for 5 hr, normal serum containing media replaced transfection media and then cells were further incubated for 19 hr before replating of cells onto collagen I or fibronectin. At the same time of replating, TGF-β1 (5 ng/ml) was treated and then incubated for 15 hr prior to harvests. The transfection efficiency was examined to be routinely 30 ~ 40% by green fluorescent protein expression (data not shown).
RESULTS

While studying possible cross-talk between integrins and TGF-β1 signaling pathways, we have observed that hepatocarcinoma Hep3B cells showed lamellipodia formation by treatment with TGF-β1 in a serum-containing normal culture conditions. The lamellipodia formation was correlated with Rac1 activation (Figure 1A). Interestingly, we also found that the TGF-β1 treatment resulted in increased levels of cyclins D1 and E, although cyclins A and B1 were reduced. However, the activity of Erk1/2, which is important for inducing cyclin D in G1 phase, was unexpectedly slightly reduced (Figure 1B). Therefore, it is possible that alternative regulatory signaling pathways, rather than Erks-mediated cyclin D1 induction, may modulate cell cycle regulators in this system, and that TGF-β1 treatment may trigger such pathways. To test this possibility, the mechanisms underlying the TGF-β1-mediated lamellipodia formation and alterations in cyclin expression were explored.

Effects of TGF-β1 on integrin expression

Integrin-mediated cell adhesion is well-known to activate the RhoA GTPase family including Rac1. Therefore, we wondered if the TGF-β1 effects were mediated by a cross-talk mechanism between the TGF-β1 and integrin signaling pathways, and if the effects are integrin signaling-dependent. First, we analyzed integrin expression levels before and after TGF-β1 treatment to cells in serum-containing culture media to see which integrin subtype might be involved in response to TGF-β1. When integrin subunits (such as α1, α2, α3, α4, α5, α6, αβ3, αβ5, β1, and β4) were examined in the absence or presence of TGF-β1 for 48 hr, the level of α2 integrin subunit, a major collagen I receptor, was increased by TGF-β1 treatment. However, the α5 subunit, a typical fibronectin receptor, was reduced (Figure 2). Other major β1-conjugating integrin subunits including another major fibronectin-binding α4 subunit were not altered (Figure 2, data not shown).
Different TGF-β1 effects on Rac1 activation and cell cyclin regulation on fibronectin

Next, we tried to examine if the TGF-β1 effects involve specific integrin signaling pathways, because lamellipodia formation and Rac1 activation usually occur through integrin-mediated cell adhesion signaling. We hypothesized that the signaling cross-talk between TGF-β1- and specific integrin type-mediated signaling pathways may lead to the Rac1 activation (thus lamellipodia formation) and increased cyclin expression. To test it, cells were replated either on collagen I or fibronectin, and at the same time TGF-β1 was added to the replating media. After a 15 hr incubation cell lysates were prepared. The incubation time was decided from preliminary experiments in which lamellipodia formation was examined over various time points from 2 to 15 h in the presence of TGF-β1 (data not shown). Interestingly, the basal (without TGF-β1 treatment) and TGF-β1-induced Rac1 activities were more obvious on fibronectin than on collagen I (Figure 3A, left). This increased Rac1 activity in cells on fibronectin with TGF-β1 treatment was correlated with lamellipodia formation (Figure 5B). Furthermore, the basal and TGF-β1-mediated cyclins E and A levels were increased in cells on fibronectin but maintained or reduced in cells on collagen I. However, levels of cyclin D1 and cyclin-dependent kinases (CDKs) 4, 6, and 2 were very similar independent of ECMs where cells were on (Figure 3A, right). The expression levels of cyclin B in cells on the both ECMs were eventually reduced and hardly detectable with TGF-β1 treatment, indicating no mitosis (Figure 3A, right). Although cyclins D and E were up-regulated by TGF-β1 in the presence of serum (Figure 1B), replated cells on fibronectin in the absence of serum showed up-regulation of cyclins E and A by TGF-β1 (Figure 3A). This inconsistency may be explained by different signaling contexts between the two experimental conditions.

Then, we have performed BrdU incorporation assay to see whether TGF-β1 treatment results in different S-phase populations in cells on collagen I or fibronectin. In the absence
of TGF-β1 treatment, cells on fibronectin showed a slightly higher rate of S-phase population (at most 20%), compare to cells on collagen I. However, cells replated on fibronectin showed further increased population of BrdU-incorporated S-phase cells with TGF-β1 treatment, compared to cells on collagen I (Figure 3C). These results suggest that traverse to S-phase was permitted in cells that encounter signals from both fibronectin receptors and TGF-β1.

The TGF-β1 effects dependent on pharmacological inhibition of c-Src family kinase

Next, we used pharmacological inhibitors to identify the effectors mediating the TGF-β1 effects. Signaling activities of Erk1/2, p38 MAPK, c-Src, FAK, and PKB/Akt, were also analyzed from cells in suspension or replated on either collagen I or fibronectin without or with TGF-β1 treatment. Erk1/2 and p38 MAPK activities were slightly reduced by TGF-β1 treatment in cells on collagen I, whereas they did not change in cells on fibronectin. However, c-Src phosphorylation at Tyr^{416} was prominent in TGF-β1-treated cells on fibronectin (Figure 4A). Meanwhile, FAK phosphorylation at Tyr^{397} and PKB/Akt phosphorylation at Ser^{473} were independent of TGF-β1 treatments and ECMs (Figure 4A). Cells replated on fibronectin showed significantly increased basal and TGF-β1-mediated Rac1 activities, compared to cells on collagen I. The TGF-β1-induced Rac1 activity was abolished by the c-Src family kinase inhibitor, PP2, but not by its negative control compound PP3 (Figure 4B). c-Src activities were also parallely regulated by the ECM types and TGF-β1 treatment. The TGF-β1 effects on cyclins E and A expression were also dependent on c-Src family kinase inhibition (Figure 4C). Interestingly, the p27^{Kip1}, but not other CKIs, levels were inversely regulated, being consistent with the TGF-β1 effects, which were also dependent on the PP2 treatment (Figure 4C). However, inhibition of Erk1/2 or p38 MAPK did not abolish the TGF-β1 effects, indicating their no involvements (data not shown).

Preferential formation of lamellipodia and focal adhesion dependent on c-Src family
kinase activity

Integrin-mediated signaling modulates lamellipodia and focal adhesion formation through actin reorganization [12]. Therefore, we tried to examine if c-Src family kinase signaling in cells on fibronectin may underlie preferential lamellipodia and focal adhesion formation even upon TGF-β1 treatment, compared to cells on collagen I. Immunofluorescence images for tyrosine phosphorylated proteins in cells on either collagen I or fibronectin in the absence or presence of TGF-β1 treatment were compared to one another. In both untreated and TGF-β1-treated cells, phospho-tyrosine staining was more prominent in focal adhesions at cell boundaries in cells on fibronectin than on collagen I (Figure 5A). Furthermore, the phospho-tyrosine staining was also inhibited by cytochalasin D treatment, which disrupts actin filament (data not shown). Again the preferential staining of phospho-tyrosine proteins at focal adhesions was dependent on c-Src family kinase inhibition (Figure 5A). In addition, TGF-β1-treated cells on fibronectin spread more with peripheral actin enrichment, with forming lamellipodia, than cells on collagen I (Figure 5B). Furthermore, the lamellipodia formation on fibronectin was inhibited by PP2, but not PP3, treatment (Figure 5B). Therefore, it is likely that TGF-β1 treatment caused different effects depending on integrin subtypes, as observed by more prominent lamellipodia formation and phospho-tyrosine staining at focal adhesions in cells on fibronectin, but not significantly on collagen I.

c-Src activity-dependent TGF-β1 effects

We next examined whether increased c-Src activity might turn on the fibronectin response after the TGF-β1 treatment in cells even on collagen I. Cells were transiently transfected with either an inactive (Y416F) or an active (Y527F) form of c-Src. Twenty four hours later, the cells were replated on collagen I in the absence or presence of TGF-β1 for 15 hr. The expression of active c-Src resulted in an increases in Rac1 activity and cyclin A expression, but a reduction in p27_Kip1 CKI level, in cells even on collagen I (Figure 6A, lanes
3 and 4). Inversely, expression of inactive c-Src in cells on fibronectin abolished the TGF-β1 effects (Figure 6A, lanes 1 and 2). Therefore, the TGF-β1 effects indeed depend on c-Src activity.

**The TGF-β1 effects in Hur7 hepatoma cells**

Next we determined whether the TGF-β1 effects can also be found in another hepatoma cell line, Hur7. As shown in figure 7, basal and TGF-β1-mediated activation of Rac1 and c-Src and expression levels of cyclins E and A were prominent in cells on fibronectin, compared to cells on collagen I. They were also dependent on c-Src family kinase activity, like Hep3B cells. Furthermore, the expression level of p27^Kip1 CKI and hypophosphorylation of retinoblastoma protein (pRb) were inversely correlated with the levels of cyclins, and dependent on c-Src family kinase activity (Figure 7B). These results indicate that G1 and S phase cyclins were up-regulated by activation of c-Src and Rac1 when TGF-β1 was treated to hepatoma cells on fibronectin. Therefore, this study represents another pathway of c-Src/Rac1/actin reorganization to regulate cell cycle progression.
DISCUSSION

In this study, we showed that TGF-β1 activated Rac1 via c-Src family kinase, and that the TGF-β1 effects correlated with enhanced expression of cyclins E and A levels and suppression of p27\(^{kip1}\) CKI in hepatoma cells replated on fibronectin, but not on collagen I, leading to another cell cycle regulation mechanism upon TGF-β1 treatment in hepatoma cells.

This current study clearly showed that TGF-β1 treatment during normal culture with serum-containing media resulted in up-regulated expression levels of cyclins D1 and E (Figure 1C), with an eventual cyclin B1 reduction. Being consistent with these observations, it was also previously shown that TGF-β1 treatment to Hep3B cells showed sustained expression of cyclin E and an eventual G2 arrest [26]. Additionally in this study, however, cells were replated on specific ECMs in the presence of TGF-β1 but in the absence of serum were analyzed. When thereby TGF-β1 pathways cross-talk with specific integrin subtype-dependent signaling, cells on fibronectin showed increased levels of cyclins E and A, compared to cells on collagen I, while cyclin D1 levels were unaffected (Figure 3). This discrepancy between serum-containing condition and no-serum containing replated-conditions as for the cyclin A expression might be presumably due to increased significance of integrin and ECM-mediated signaling in the replated conditions. That is, cells replated on fibronectin could have signaling activities initiated only from TGF-β1 and fibronectin-integrin interaction mediated signaling, whereas TGF-β1-treated cells within serum-containing media might have diverse signaling activities mediated by serum components. The key effectors downstream of integrin receptors include c-Src and Rac1 and etc, leading to the activation of diverse intracellular signaling and actin reorganization (e.g., lamellipodia formation) and the regulation of cell cycle regulators [4, 5, 8, 9, 10, 11, 12]. Furthermore, in this study, the increased expression levels of cyclins in the presence of TGF-β1 correlated with c-Src activation and lamellipodia formation in cells replated on only specific ECM
substrates. These results indicate specificity in signal cross-talks between integrin- and TGF-β1-mediated pathways, resulting in differential signaling activities depending on ECMs where cells are on. It is well known that not only integrin-mediated signaling but also mitogenic signaling activities is required for cell cycle progression through regulation of cyclin levels, because normal epithelial cells do not divide in suspension where no cell adhesion signaling is available [4, 27].

It was previously shown that TGF-β1 could activate Rac1, when cells were treated with TGF-β1 for a relatively shorter time of 2.5 min or 60 min [28, 29]. In this current study, Rac1 activation was shown 15 hr after TGF-β1 treatment, when lamellipodia formation and S phase cell accumulation was significant in cells on fibronectin. In another previous study, the activity of CDC42 and RhoA by TGF-β1 showed two peaks, one for around 5 ~ 15 min and the other around 6 ~ 12 hr after a TGF-β1 treatment [30]. We show here that GTPases including Rac1 is also downstream effector of TGF-β1 signaling after prolong TGF-β1 treatment in hepatoma cells.

On the other hand, it was also previously shown that TGF-β1 influenced c-Src activity either negatively or positively depending on the cell type and/or the nature of its treatment. HaCaT human keratinocytes, MDCK kidney epithelial cells, and Mahalavu hepatoma cells, showed increases in c-Src activity [31, 32], when TGF-β1 was treated for 15 min, unlike this current study where cells were treated for 15 hr. Meanwhile v-Src transformed rat fibroblasts and HepG2 hepatoma and PC3 human prostate carcinoma cells showed reduced c-Src activity and protein abundance with TGF-β1 treatment [32, 33]. Therefore, the effects of TGF-β1 on c-Src may also be dependent on the cell types and/or signal contexts under which TGF-β1 treatment occurred.

In this study, c-Src appears to be upstream of Rac1, cyclins, and p27Kip1 CKI, since its pharmacological inhibition of c-Src family kinase abolished the TGF-β1 effects on Rac1
activation, lamellipodia formation, increased cyclins E and A levels, and a decreased p27^{Kip1} CKI level in cells on fibronectin. In addition, expression of constitutively active c-Src in cells on collagen I increased basal and TGF-β1-mediated levels of Rac1 activity, cyclin A expression and suppressed p27^{Kip1} levels. Such a relationship between c-Src and Rac1 was recently reported by showing that c-Src phosphorylates guanine nucleotide exchange factor (GEF) for Rac1 such as Tiam1 and Vav2 to regulate Rac1 activity in NIH3T3 cells [34].

It is interesting to note that the TGF-β1 treatment decreased the integrin α5 subunit (a typical fibronectin receptor), and increased integrin α2 subunit (a major collagen I receptor), while there were no alterations in other types tested, such as another major fibronectin-binding integrin α4 as well as other several β1-binding subtypes (Figure 2, data not shown). However, basal and TGF-β1-mediated activations of c-Src family kinase and Rac1 and increases in cyclins E and A expression in cells on fibronectin were preferentially and significantly higher than those in cells on collagen I. In addition, integrin-mediated signaling monitored by phospho-tyrosine proteins or actin immunostainings was more prominent in cells on fibronectin than in cells on collagen I. Such prominent signaling may presumably lead to the preferential TGF-β1 effects on fibronectin, even with decreased levels of integrin α5. An explanation of this paradox may involve signaling efficiency with a lower threshold in TGF-β1-treated cells on fibronectin, although it remains to be explored. That is, even a reduced level (but not to null) of integrin α5 could be enough for efficient transduction (via a lower threshold) of signal cross-talks with TGF-β1 pathway toward c-Src and Rac1, whereas signal cross-talks via integrin α2 or collagen receptors could not presumably due to a higher threshold. In addition to this study, there is previous evidence that signaling activity upon growth factor treatment in cells on fibronectin (presumably via the α5 integrin subunit) is more efficient than that in cells on collagen I or laminin (presumably via the α2 integrin subunit) [17, 24, 25]. On the other hand, it may also be possible that
TGF-β1 caused a signaling shift from by integrin α5-mediated fibronectin binding to by adhesion through other fibronectin-binding integrin types, although another major fibronectin-binding integrin α4 was not significantly regulated by the TGF-β1 treatment (Figure 2).

Taken together, this study showed that TGF-β1 treatment on hepatoma cells resulted in preferential Rac1-mediated lamellipodia formation, expression of cyclins E and A, and suppression of p27\(^{kip1}\) CKI level in cells on fibronectin, but not on collagen I. All of these TGF-β1 effects were dependent on c-Src activity. These observations may indicate that another putative mechanism involving c-Src/Rac1/actin organization to regulate cell cycle regulators, when TGF-β1 pathway encountered a specific integrin-mediated signal pathway in hepatoma cells.
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LEGENDS TO FIGURES

Figure 1.  **TGF-β1-mediated morphological changes and regulation of Rac1 activity and cyclins expression in Hep3B cells.** (A, upper) Phase-contrast images of Hep3B cells untreated (control) or treated with TGF-β1. TGF-β1 (5 ng/ml) was treated by adding directly to serum-containing culture media for 48 hr prior to taking images. (A, lower) TGF-β1-mediated Rac1 activation in Hep3B cells. Cells in normal culture media were treated with TGF-β1 prior to cell harvests. Lysates prepared with a modified RIPA buffer (without the SDS detergent) were used for the Rac1 assay by *in vitro* GST-PAK1 (76-150) pull-down, as described in Material and methods. Controls show equal amounts of GST-PAK1 fusion proteins or Rac1. Data shown are representative from several independent experiments. (B) Regulation of Erk1/2 activity and cyclins levels by TGF-β1 treatments to Hep3B cells. Cells were treated with indicated amounts of TGF-β1 for 48 hr in normal culture media before lysate preparation. Equal amount of proteins in the lysates were separated by SDS-PAGE electrophoresis and blotted for antibodies against indicated molecules. Data shown are representative from three isolated experiments.

Figure 2.  **TGF-β1-treatment increases α2 integrin subunit, but reduces α5 integrin subunit.** Integrin expression levels were analyzed by flow cytometric measurements without or with TGF-β1 treatment. TGF-β1 (5 ng/ml) was directly added to normal culture media, and the treatment lasted for 48 hr until cell harvests for flow cytometric measurement. Data show histograms for integrin α2 (top), α5 (middle), or α4 (bottom) subunits. Histograms for a negative control (C, without primary antibody), untreated (Un) or treated with TGF-β1 (T) were included.

Figure 3.  **Basal and TGF-β1-mediated increases in Rac1 activity, cyclins E and A, and**
**S-phase Hep3B cells on fibronectin.**  (A) Differential effects mediated by TGF-β1 on Rac1 activity and cyclins expression in cells fibronectin, compared to cells on collagen I. Cells were trypsinized, washed twice with the replating media of RPMI-1640 plus 1% BSA, kept in suspension for 1 hr at 37°C with rocking, and then replated either on collagen I (Coll) or fibronectin (Fn), in the absence or presence of TGF-β1 treatment for 15 hr at 5 ng/ml. Lysates were prepared 15 h after the replating of cells, as explained in Materials and methods, and used for immunoblottings against indicated molecules. Data shown are representative from several isolated experiments. (B) Preferential accumulation of S-phase cells on fibronectin after TGF-β1 treatment for 15h. Replating of cells on ECM-precoated coverslips and incorporation of BrdU were performed, as explained in Materials and methods. Then staining of BrdU-positive cells was performed with anti-BrdU antibody and then FITC-conjugated secondary antibody, prior to visualization with a fluorescence microscope. Data shown are representative from two different experiments, in which images of 5 independent areas were recorded and BrdU-positive cells were counted for mean value calculations. Data are shown as mean ± standard deviation (SD).

**Figure 4.**  Basal and TGF-β1-mediated Rac1 activation, cyclins E and A expression and p27Kip1 CKI suppression appear to involve c-Src family kinase activity. Cells were kept in suspension or replated on collagen type I (Coll) or fibronectin (Fn) in the absence or presence of TGF-β1 treatment (5 ng/ml, 15 hr). Pretreatments of c-Src inhibitor (PP2, 10 μM) or its negative control compound PP3 (10 μM) were performed, by adding directly to the replating media 30 min prior to replating of cells on fibronectin. Cell lysates were used for Western blots for indicated molecules or Rac1 assay. Data shown are representative from at least 3 independent experiments. (A) Signaling activities in cells either in suspension (Sus) or replated on collagen I (Coll) or fibronectin (Fn) without or with TGF-β1 (5 ng/ml).
treatment for 15 hr. (B) Basal and TGF-β1-mediated activation of c-Src and Rac1 on fibronectin were abolished by inhibition of c-Src family kinase activity. (C) Regulation of basal and TGF-β1-mediated increases in cyclins E and A and a decrease in p27^Kip1 expression, but not other CKIs, involves c-Src family kinase activity.

**Figure 5.** Hep3B cells on fibronectin showed more prominent basal and TGF-β1-mediated phospho-tyrosine staining and lamellipodia formation than on collagen I. Cells were replated onto coverslips pre-coated with either collagen I (Coll) or fibronectin (Fn) in the absence (untreated) or presence of TGF-β1 treatment at 5 ng/ml (TGF-β1) for 15 hr, prior to immunofluorescence microscopy using anti-phosphotyrosine antibody and FITC-conjugated anti-mouse IgG (A) or phalloidin-conjugated with Rhodamine (B). c-Src family kinase inhibitor (PP2) or its negative control compound (PP3) was pretreated 30 min before the replating. Shown images are representative from at least 5 isolated fields in each immunostaining.

**Figure 6.** c-Src activity is required for regulation of basal and TGF-β1-mediated Rac1 activity, cyclin A expression, and p27^Kip1 CKI suppression. Cells were transiently transfected with cDNAs for inactive (Y416F) or active (Y527F) c-Src. Twenty four hour posttransfection, cells were replated on collagen I. At the same time of replating, TGF-β1 was added directly to media and incubation lasted for 15 hr. Cell lysates were used either for Rac1 pull-down assay or immunoblotting with antibodies against the indicated molecules. Upon ectopic expression of active c-Src, endogenous c-Src was relatively activated probably due to c-Src cross-activation. Data shown are representative from at least two different experiments.
Figure 7. The differential basal and TGF-β1-mediated effects in Hur7 hepatoma cells. Hur7 cells were manipulated as Hep3B explained above, for replating and treatments of TGF-β1, PP2, and PP3. Lysates prepared as explained above were used for Western blots for the indicated molecules. Data shown are representative from two independent experiments. (A) The preferential activation of Rac1 in cells on fibronectin over cells in collagen I depend on c-Src family kinase activity. (B) The differential effects of TGF-β1 treatment to Hur7 cells on fibronectin on cell cycle regulators.
Figure 1, Hwang-Phill Kim, et al

(A) TGF-β1 (ng/ml, 2d) pull-down lysate

Control TGF-β1

GTP-Rac1
GST-PAK1
Total Rac1

(B) p-Erk1/2 Erk1/2 Cyclin D1 Cyclin E Cyclin A Cyclin B1 β-tubulin

p-Erk1/2 Erk1/2 Cyclin D1 Cyclin E Cyclin A Cyclin B1 β-tubulin

TGF-β1 (ng/ml, 2d) 0 5 20

Hep 3B
Figure 2, Hwang-Phill Kim, et al

![Diagram of C, Un, and T for α2, α5, α4 events]
Figure 3, Hwang-Phill Kim, et al.

(A)

![Western blot images showing Rac1-GTP, Total Rac1, Cdk4, Cdk6, Cdk2, Cyclin D1, Cyclin E, Cyclin A, and Cyclin B1 levels with or without TGF-β1 stimulation.](image)

(B)

![Bar graph showing BrdU-positive cells (%) with or without TGF-β1 stimulation.](image)
Figure 4, Hwang-Phill Kim, et al

(A) p-Erk1/2
Erk1/2
p-p38
p38
pY416c-Src
c-Src
pY397FAK
FAK
pS473PKB
PKB
TGF-β1 ++ – – + – +
Sus Coll Fn

(B) GTP-Rac1
Total Rac1
pY416c-Src
c-Src
Inhibitor – – – PP2 PP3
TGF-β1 ++ – + + +
Coll Fn
Figure 4, Hwang-Phill Kim, et al

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<thead>
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<td>p21Cip1/Waf</td>
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Inhibitor  
TGF-β1: +
Coll: +
Fn: +
PP2: +
Figure 5, Hwang-Phill Kim, et al

(B)
Figure 6, Hwang-Phill Kim, et al

- pY416c-Src
- c-Src
- Rac1-GTP
- Cyclin A
- p27Kip1
- p16INK4a
- p21Cip1/Waf

TGF-β1

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<td>Y527F</td>
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Collagen I
Figure 7, Hwang-Phill Kim, et al

(A)  
- GTP-Rac1  
- Total Rac1  
- pY416c-Src  
- c-Src

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Hur 7

(B)  
- Cyclin D1  
- Cyclin E  
- Cyclin A  
- p27  
- pRb  
- α-Tubulin

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Hur 7