# Hic-5 Promotes the Hypertrophic Scar Myofibroblast Phenotype by Regulating the TGF-β1 Autocrine Loop

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Following severe traumatic or thermal injury to the dermis, hypertrophic scars (HTSs) often develop in humans. These scar fibroblasts (hypertrophic scar fibroblasts (HTSFs)) retain the myofibroblast phenotype persistently, rather than transiently as in acute wounds. These pathogenic myofibroblasts constitutively express smooth-muscle cell α-actin (SMAA), deposit an excessive amount of extracellular matrix (ECM) proteins, are highly contractile, and stably display large focal adhesions. Increasing evidence supports a mechanism in which autocrine production and activation of transforming growth factor-β1 (TGF-β1) are required to maintain the pathogenic myofibroblast phenotype. We recently reported that Hic-5, a focal adhesion protein that is upregulated by TGF-β1, is expressed persistently in HTSF compared to normal adult fibroblasts (NADFs). We now find that Hic-5 is an important regulator of the constitutive myofibroblast phenotype in HTSFs. Silencing the expression of Hic-5 in HTSFs with specific siRNAs dramatically reduces TGF-β1 production, decreases the generation of supermature focal adhesions reduces expression of SMAA and decreases collagen contraction and ECM synthesis. Our findings demonstrate that Hic-5 is an essential component of the mechanism regulating autocrine production of TGF-β1 and the resulting pathogenic myofibroblast phenotype.

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## INTRODUCTION

Fibrotic diseases, whether in the skin or other organs, are a pathogenic form of scar formation in which excessive deposition and contraction of extracellular matrix proteins can dramatically reduce organ function and compromise cosmetic appearance (Wynn, 2007). Scar formation, whether normal or pathogenic, is driven by myofibroblasts, a cell type differentiated from quiescent fibroblasts in a process that requires transforming growth factor-β1 (TGF-β1) and mechanical stress (Tomasek *et al.*, 2002; Hinz, 2007). While myofibroblast differentiation during normal acute wound healing is temporally limited, in fibrotic settings these myofibroblasts persist in relatively high numbers for long periods following injury. Hypertrophic scars (HTSs), one form of cutaneous fibrotic disorder, develop after thermal or traumatic injury to the deep dermis (Tuan and Nichter, 1998). Histological examination of HTSs shows distinct nodules containing fine collagen fibers and an excessive number of smooth-muscle cell α-actin (SMAA)-positive myofibroblasts (hypertrophic scar fibroblasts (HTSFs)) (Ehrlich *et al.*, 1994). HTSF persistence is thought to occur as a consequence of the autocrine production and activation of TGF-β1 (Scott *et al.*, 2000; Tredget *et al.*, 2000).

This putative mechanism for TGF-β1 is likely important in a broader spectrum of fibrotic disorders (Blobe *et al.*, 2000). However, the mechanism through which the TGF-β1 autocrine loop controls the pathogenic myofibroblast phenotype remains largely unresolved. We recently reported that cultured fibroblasts derived from HTSFs stably exhibit an autocrine TGF-β1 loop that promotes assembly and maintenance of large focal adhesions, structures mediating adhesion, collagen contraction and serving as intracellular signaling centers (Dabiri *et al.*, 2006). An intriguing potential connection between focal adhesions and TGF-β1 regulation led us to hypothesize that Hic-5 was important in the HTSF phenotype.

Hic-5 is a TGF-β and $H_2O_2$-inducible focal adhesion protein that serves as a transcription cofactor for $p21^{Cip1}$, c-fos, PPAR-γ, and a coactivator for androgen receptors and glucocorticoid receptors (Shibanuma *et al.*, 1997, 2003; Thomas *et al.*, 1999; Yang *et al.*, 2000; Kim-Kaneyama *et al.*, 2002). Hic-5 is unique among focal adhesion proteins because it can shuttle between focal adhesions and the nucleus in response to oxidants such as $H_2O_2$ (Shibanuma *et al.*, 2003). Given these properties, we recently reported that Hic-5 is expressed constitutively by HTSFs and induced by TGF-β1 in normal adult dermal fibroblasts (NADFs). Moreover, Hic-5 is both necessary and sufficient to mediate TGF-β1-regulated cell-cycle progression in NADFs and HTSFs (Dabiri *et al.*, 2008).

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*Abbreviations: FN, fibronectin; GFP, green fluorescent protein; HTS, hypertrophic scar; HTSF, hypertrophic scar fibroblast; NADF, normal adult human dermal fibroblasts; SMAA, smooth-muscle cell α-actin; TGF-β1, transforming growth factor-β1*

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In this study, we directly tested whether or not Hic-5 was required for the generation of the myofibroblast phenotype by analyzing SMAA expression, focal adhesion maturation, extracellular matrix (ECM) synthesis, and collagen contraction in NADFs. To test the extent to which Hic-5 was required for persistence of the HTSF phenotype, we employed genetic silencing, finding that Hic-5 ''knockdown'' with RNA interference reversed the myofibroblast phenotype of HTSFs to that of a resting fibroblast phenotype. We also found that Hic-5 was required to maintain the autocrine loop of TGF-β1 in HTSFs by regulating TGF-β1 production. Thus, Hic-5 is upregulated by TGF-β1 and, in turn, is required for production of TGF-β1 in a feed-forward mechanism in which TGF-β1 levels remain constitutively high, thereby maintaining the pathogenic myofibroblast phenotype.

## RESULTS

### Hic-5 is necessary for maintenance of supermature focal adhesions in HTSFs

HTSFs persistently express a higher percentage of supermature focal adhesions ($\geqslant 6\,\mu m^2$) per cell compared with NADFs. The persistence of these focal adhesions in HTSFs was maintained by elaboration of an autocrine loop generating active TGF-β1, since inhibition of TGF-β1 significantly decreased the area of focal adhesions in HTSFs (Dabiri *et al.*, 2006). We sought to determine how TGF-β1 maintained these supermature focal adhesions in HTSFs. To do so, either Hic-5 or control short interference RNAs (siRNAs) were transfected into either HTSFs or NADFs. Cells were then trypsinized and cultured in the presence or absence of TGF-β1 ($10\,ng\,ml^{-1}$) for 5 days. These cells were fixed, stained with anti-vinculin, and the areas of focal adhesion were measured (Figure 1). Western blot analysis of Hic-5 was also performed to determine whether or not the siRNAs to Hic-5 were effective in decreasing Hic-5 levels in NADFs and HTSFs (Figure 2b). We also observed by immunofluorescence that greater than 90% of the cells transfected with Hic-5 RNA interference lost Hic-5 staining (data not shown). We found that 20% of the focal adhesions in HTSFs measured greater than $6\,\mu m^2$ compared with approximately 7% seen in NADFs using vinculin immunofluorescent staining. The addition of TGF-β1 to NADFs resulted in a marked increase in the percentage of focal adhesions measuring greater than $6\,\mu m^2$. Upon genetic silencing of Hic-5 in HTSFs, a significant decrease in the percentage of focal adhesions measuring greater than $6\,\mu m^2$ was observed. Since TGF-β1 regulates the size of focal adhesions, we wanted to determine whether or not Hic-5 was necessary for this TGF-β1-dependent effect. In the absence of Hic-5, addition of TGF-β1 increased the size of focal adhesions to levels comparable to that of control siRNA in the presence of TGF-β1 in NADFs. Interestingly, in the absence of Hic-5, but with the addition of exogenous TGF-β1 to HTSFs, focal adhesion areas were restored to those of control HTSFs. Although decreasing the area of focal adhesions, genetic silencing of Hic-5 in HTSFs did not alter the overall levels of vinculin or paxillin expressed (data not shown). These results indicated that for both NADFs and HTSFs, Hic-5 was not necessary for the TGF-β1-mediated generation of supermature focal adhesions. However in HTSFs, Hic-5 was important for the constitutive presence of supermature focal adhesions.

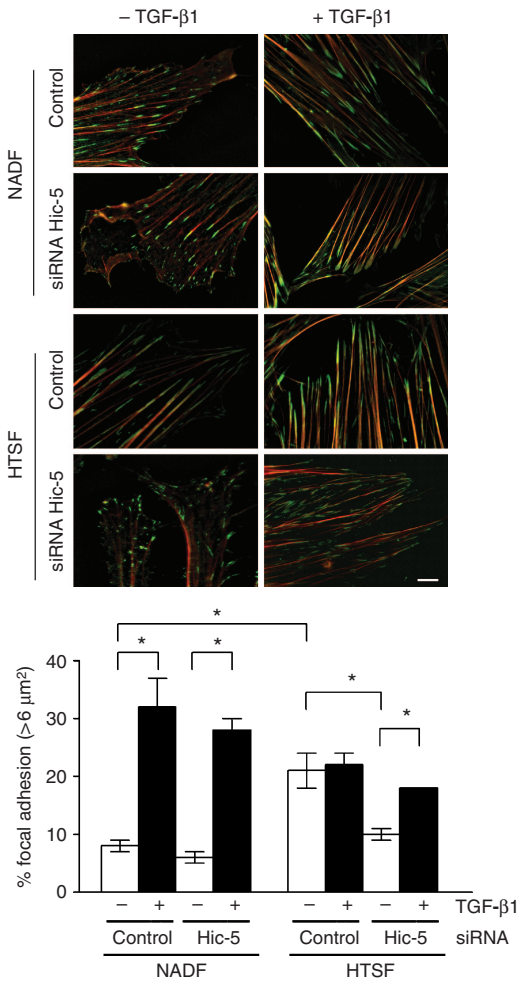


**Figure 1. Hic-5 helps to maintain the persistence, but not generation, of supermature focal adhesions.** Cells were transfected (5 days) with either siRNA to Hic-5 or scrambled control (see Materials and Methods). Cells were then trypsinized and replated in medium containing serum for 18 hours, washed, and cultured in serum-free medium either with or without addition of TGF-β1 (5 days). Cells were then stained for vinculin (green) and stress fibers (red). Percent focal adhesions greater than $6\,\mu m^2$ were calculated as the ratio between the total number of focal adhesions greater than $6\,\mu m^2$ and the total number of focal adhesions multiplied by 100. $^*P<0.005$, $n=3$. Bar = 10 μm.

### Hic-5 is important for maintenance of SMAA expression but not generation of SMAA by TGF-β1

TGF-β1 is important in the transition from fibroblast to myofibroblast, and in culture this differentiation occurs over the course of 5 days (Desmoulière *et al.*, 1993). Since we found earlier that Hic-5 is regulated by TGF-β1, and that Hic-5 is important for the constitutive presence of supermature focal adhesions in HTSFs, it was of interest to determine whether Hic-5 levels are maintained during the course of the 5-day transition from a fibroblast to differentiated myofibroblast. Either NADFs or HTSFs were cultured in serum-free media for 5 days with or without addition of TGF-β1 ($10\,ng\,ml^{-1}$) or anti-TGF-β1 ($20\,\mu g\,ml^{-1}$). We found that

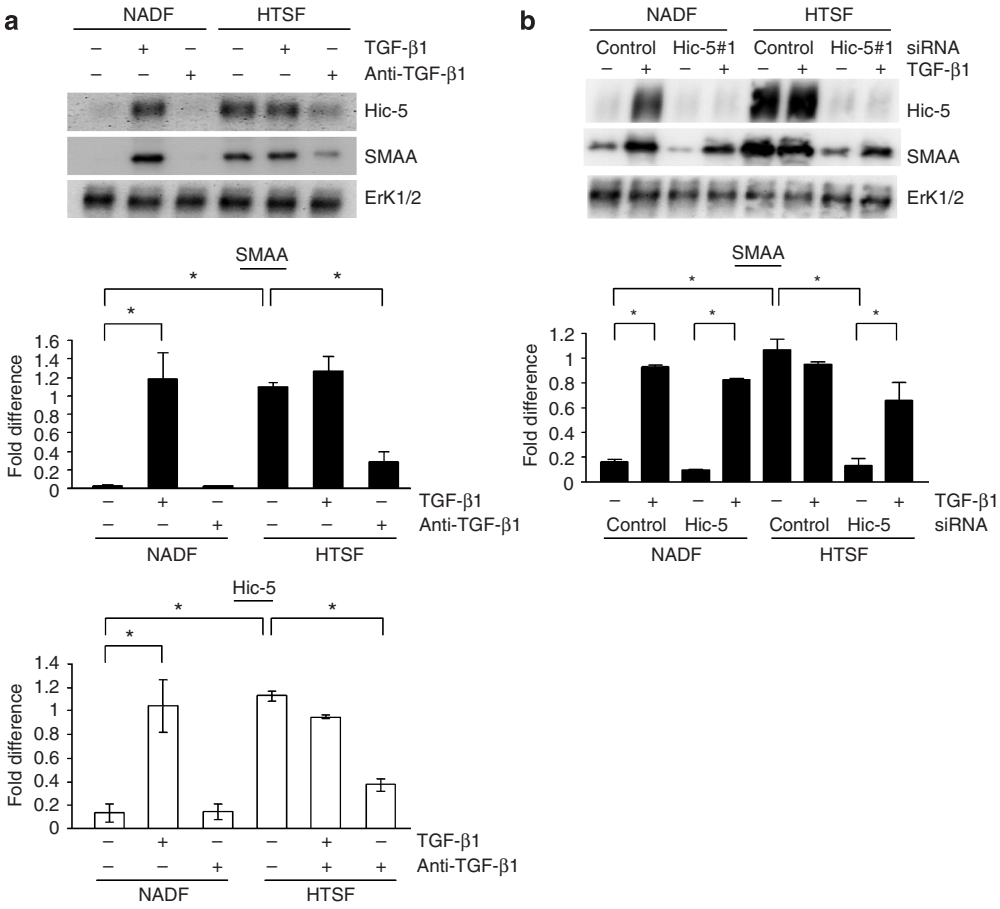


**Figure 2. Hic-5 maintains the expression of SMAA in HTSFs, but TGF-β1 is necessary for initiating fibroblast differentiation.** (**a**) Cells without siRNA transfection were trypsinized and replated (18 hours) in serum-containing medium, washed, and cultured (5 days) in serum-free medium either with or without addition of TGF-β1 (10 ng ml$^{-1}$) or anti-TGF-β1 (20 μg ml$^{-1}$). Cells were then lysed and directly resolved by SDS–PAGE (10%) and analyzed by western blotting. The intensity of each band was normalized, first for differences in protein loading using Erk1/2, and then to HTSFs for each condition. Data from replicate experiments were pooled and presented relative to HTSF control ($n=4$). (**b**) Cells were transfected (5 days) as described under Materials and Methods. Cells were then trypsinized and replated in medium containing serum (18 hours), washed, and cultured (5 days) in serum-free medium either with or without TGF-β (10 ng ml$^{-1}$). Cell lysates were plated, applied to SDS–PAGE gels (10%), analyzed by western blotting, and quantitated as described above. *$P<0.005$, $n=3$.

HTSFs were constitutively myofibroblastic, expressing SMAA persistently; Hic-5 expression was also maintained in HTSFs during 5 days in serum-free media (Figure 2a). With addition of TGF-β for 5 days, both SMAA and Hic-5 were upregulated in NADFs to levels comparable to HTSF control. Addition of anti-TGF-β1 to HTSF cultures decreased the expression of both Hic-5 and SMAA (Figure 2a), indicating that the autocrine loop of TGF-β1 maintained the expression levels of both SMAA and Hic-5. Since Hic-5 regulated the maintenance, but not generation, of supermature focal adhesions in HTSFs, and as there is a link between SMAA, intracellular tension, and focal adhesion development (Hinz, 2007), we next determined whether inhibition of Hic-5 regulated SMAA expression. Either Hic-5 or control siRNAs were transfected into HTSFs and NADFs for 5 days. Cells were trypsinized and replated in serum-free medium in the presence or absence of TGF-β1 (10 ng ml$^{-1}$) for 5 days. We found that genetic silencing of Hic-5 decreased the expression of SMAA in HTSFs. In NADFs or HTSFs treated with Hic-5 siRNA, TGF-β1 was still able to increase the expression of SMAA to levels as high as those of HTSF controls (Figure 2b). Cells were also immunostained for SMAA to ensure that the differences in protein expression levels observed by western blotting (Figure 2b) correlated with SMAA assembled into stress fibers (data not shown). We found that approximately 80% of the HTSFs were SMAA-positive and that inhibition of Hic-5 decreased the percentage of SMAA-positive cells by eightfold to 10%. The addition of TGF-β1 to HTSFs in which Hic-5 was genetically ablated resulted in a fivefold increase in the percentage of SMAA-positive cells. In NADFs, addition of TGF-β1 increased the percentage of SMAA-positive cells by sevenfold and in the absence of Hic-5, addition of TGF-β1 was still able to increase SMAA to levels comparable to those of NADF control siRNA. These results suggest a mechanism in which Hic-5 is required to maintain persistent SMAA expression in HTSFs, but is not required to initiate differentiation of fibroblasts to myofibroblasts.

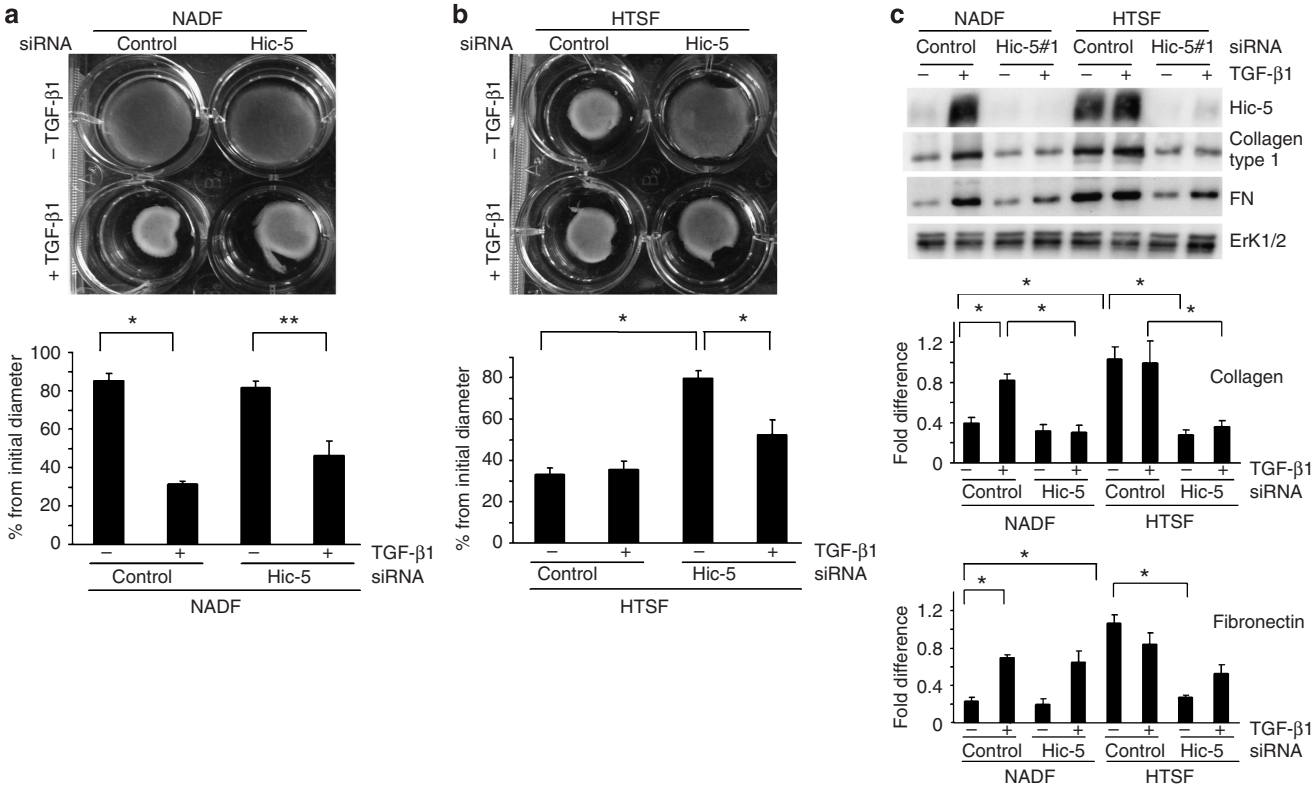


**Figure 3. Hic-5 maintains the ability of HTSFs to contract collagen and produce ECM proteins. Cells were transfected (5 days) with siRNA as described under Materials and Methods.** (**a**) NADFs or (**b**) HTSFs were then trypsinized and cultured in a collagen lattice, rimmed, and covered with serum-free medium for 3 days. The percent from initial diameter was measured as the ratio between the decrease in diameter and the original diameter at day 3 multiplied by 100. $^{*}P<0.005$, $^{**}P<0.05$, $n=3$. (**c**) Cells were transfected (5 days) with siRNA (see Materials and Methods) and cultured as described in Figure 2b. Cells were lysed and lysates were resolved by SDS–PAGE (8%) and analyzed by western blotting. $^{*}P<0.005$, $n=3$.

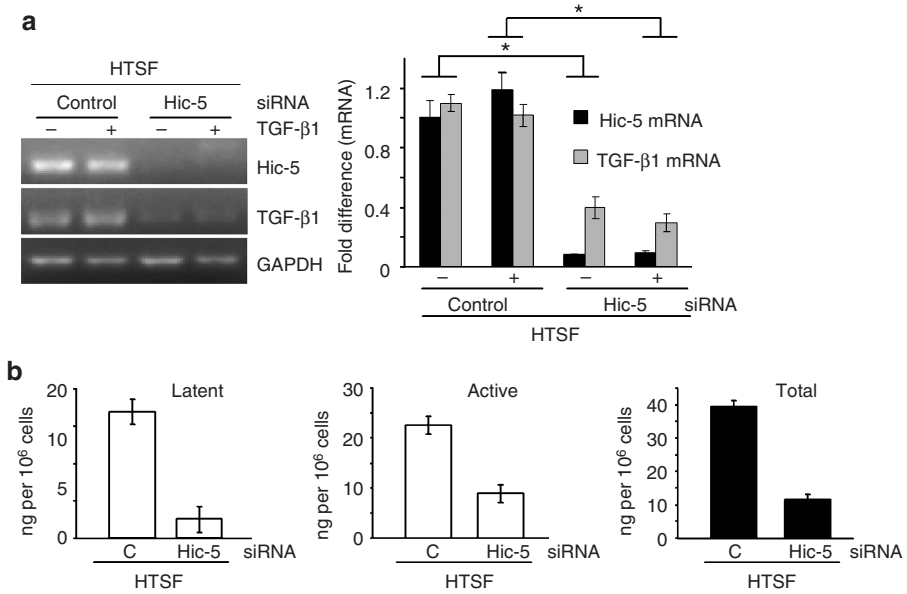


**Figure 4. siRNA Hic-5 disrupts the autocrine loop of TGF-β1 in HTSFs.** Cells were transfected (5 days) with siRNA (see Materials and Methods) and cultured as described in Figure 2b. (**a**) Total RNA was isolated from HTSFs and reverse transcriptase-PCR was performed to compare TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase mRNA levels relative to untreated HTSFs, or (**b**) conditioned medium was collected from the samples without addition of TGF-β1 and sandwich ELISA using antibody reactive with active TGF-β1 was performed on conditioned media to measure the amount of active TGF-β1 and the amount of total TGF-β1 (acid-treated to activate latent-TGF-β1). Amounts of latent-TGF-β1 were determined by subtracting the levels of active TGF-β1 from total TGF-β1. $^{*}P<0.005$, $n=3$.

**Hic-5 regulates the expression of ECM proteins and modulates the ability of HTSFs to contract collagen**

SMAA-positive fibroblasts are important in wound closure due to their ability to contract the matrix, a function that has been modeled *in vitro* using collagen contraction assays (Grinnell, 1994). Since knocking down the expression of Hic-5 decreased SMAA levels, we wanted to determine whether or not inhibition of Hic-5 disrupted the ability of HTSFs to contract collagen. Either Hic-5 or control siRNA were transfected into either HTSFs or NADFs. Cells were trypsinized and cultured in serum-free medium in the presence or absence of TGF-β1 (10 ng ml$^{-1}$) for 5 days. Cells were then cultured in a floating collagen lattice for 3 days (Figures 3a and b) or in an attached collagen lattice (data not shown). Western blots were performed on cell lysates to confirm that siRNA to Hic-5 was effective in decreasing Hic-5 levels in NADFs and HTSFs (Figure 3c). We found that HTSFs contracted collagen more extensively (threefold) compared with NADF in a floating collagen lattice (Figures 3a and b) and in an attached collagen lattice (not shown). As with the floating collagen lattice, inhibiting the autocrine loop of TGF-β1 in HTSFs significantly decreased their ability to contract an attached collagen lattice compared with the control (Dabiri *et al.*, 2006). The addition of TGF-β1 to NADFs decreased the collagen diameter by threefold, comparable (Figure 3a) to the contraction observed with HTSFs treated with control siRNA (Figure 3b). When Hic-5 was silenced in NADFs in the presence of TGF-β1, they were still able to contract collagen (Figure 3a). Knocking down the expression of Hic-5 resulted in a decrease in the ability of HTSFs to contract collagen (Figure 3b). However, addition of TGF-β1 rescued the ability of HTSFs to contract collagen (Figure 3b). These data indicate that Hic-5 is not necessary to support the hypercontractile state of HTSFs provided sufficient active TGF-β is present.

TGF-β1 induces the expression of collagen and fibronectin (FN) in fibroblasts and Hic-5 overexpression increases the mRNA levels of certain ECM proteins (Shibanuma *et al.*, 1997). We next tested whether or not Hic-5 regulates the expression of FN and collagen type I in HTSFs and/or NADFs (Figure 3c). Either Hic-5 or control siRNA was transfected into HTSFs or NADFs and cells were trypsinized and cultured in the presence or absence of TGF-β1 (10 ng ml$^{-1}$) for 5 days. We found that compared with NADFs, HTSFs expressed approximately threefold more collagen and total FN. These experiments were conducted in serum-free medium; thus, the total FN levels measured comprised cellular FNs; blotting with an antibody to the EIIIA (ED-A) FN segment confirmed its presence, characteristic of cellular FN (not shown). Inhibiting the autocrine loop of TGF-β1 in HTSFs significantly decreased the expression of both collagen and total FN (data not shown). The addition of TGF-β1 significantly increased the expression of these ECM proteins in NADFs to levels comparable to those in HTSFs. Interestingly, treatment of HTSFs with Hic-5 siRNAs resulted in a decrease in collagen and total FN expression. The addition of TGF-β1 to NADFs in the absence of Hic-5 did not increase the expression of collagen, but did significantly increase the expression of total FN, albeit not to control levels, indicating that Hic-5 is required for the TGF-β1 induction of collagen (Figure 3c). However, partial restoration of FN expression by TGF-β1 in the absence of Hic-5 indicates that FN expression may be regulated by two pathways: one dependent upon, and the other independent of, Hic-5.

These experiments tested the role of Hic-5 in maintaining and establishing the HTSF phenotype. We were also interested in testing whether or not Hic-5 was sufficient to cause differentiation of fibroblasts to myofibroblasts without the presence of TGF-β1. To test this, NADFs were infected with adenoviral constructs expressing either green fluorescent protein (GFP) control or GFP-Hic-5 for 5 days in serum-free medium, as described previously (Dabiri *et al.*, 2008). We found that NADFs in which Hic-5 was overexpressed were unable to increase either size of focal adhesions or expression of SMAA; neither did they induce contraction of collagen nor increased the expression of collagen or FN (not shown). These results indicated that Hic-5 was not sufficient to drive the fibroblast to myofibroblast differentiation. However, Hic-5 was necessary to maintain HTSFs in a constitutive myofibroblastic phenotype.

**Hic-5 regulates the autocrine loop of TGF-β1 in HTSFs**

When we silenced Hic-5 with specific siRNA in HTSFs, these pathogenic cells reverted to a lesser, differentiated fibroblast phenotype, and addition of TGF-β1 caused differentiation of these resting fibroblasts back to a myofibroblast phenotype, suggesting that Hic-5 could be regulating the autocrine loop of TGF-β1 in HTSFs. Either Hic-5 or control siRNAs were transfected into HTSFs; cells were then trypsinized and replated in serum-free medium for 5 days with or without addition of TGF-β1 (10 ng ml$^{-1}$). Conditioned medium was then collected from cells not treated with exogenous TGF-β1. RNA was isolated from all cells and reverse transcriptase-PCR was performed to compare the mRNA levels of TGF-β1. We found that knocking down Hic-5 resulted in decreased TGF-β1 mRNA levels (Figure 4). The addition of exogenous TGF-β1 did not increase TGF-β1's own mRNA expression (Figure 4a). To test TGF-β1 protein levels, conditioned medium was assayed using a sandwich ELISA. We found that the amount of both active and total (obtained after acid activation of the conditioned media) was decreased in the siRNA Hic-5-transfected HTSFs (Figure 4b). Our findings demonstrated that autocrine production of TGF-β1 was regulated by Hic-5. We also tested whether or not overexpressing Hic-5 had any effect on TGF-β1 production, secretion, or activation. NADFs were transduced with GFP-Hic-5 or GFP-control for 5 days in serum free conditions, and RNA and conditioned medium was isolated after the fifth day. Overexpressing Hic-5 increased the mRNA levels of TGF-β1 and the amount of secreted latent TGF-β1, but it did not increase the amount of active TGF-β1 (data not shown). These results demonstrate that Hic-5 is both necessary and sufficient to regulate the production, but not activation, of TGF-β1.

We conclude that Hic-5 is necessary for maintaining the perpetual myofibroblast phenotype seen in HTSFs by virtue of its regulation of endogenous TGF-β1 production. However, it

is not sufficient to drive fibroblast differentiation to myofibroblast in NADFs. These data identify Hic-5 as a critical component of the mechanism through which the autocrine loop in HTSFs is maintained resulting in the pathogenesis of HTSs.

## DISCUSSION

TGF-β1 is a critical regulator of myofibroblast generation and function, and we, and others, postulate that myofibroblast's persistence in fibrotic lesions results from the self-production and activation of TGF-β1 through an ''autocrine loop'' (Dabiri *et al.*, 2006, 2008; Hinz, 2007). Tredget *et al.* (2000) reported that hypertrophic scar tissues and fibroblasts produce more TGF-β1 mRNA and protein than normal skin and cells. We determined that autocrine production of TGF-β1 in HTSFs resulted in constitutively larger focal adhesion compared with in NADFs (Dabiri *et al.*, 2006). These larger adhesions mediated tighter binding to plasma FN and enhanced collagen contraction to a greater extent in HTSFs compared with that in NADFs. Recently, we reported that persistent TGF-β1 expression through an autocrine loop slowed HTSF cell proliferation (Dabiri *et al.*, 2008).

In this study, we identify a critical element, the focal adhesion protein Hic-5, in the autocrine production of TGF-β1, which supports the persistent myofibroblast phenotype seen in HTSFs. Genetic silencing with Hic-5 results in attenuation of the autocrine production of TGF-β1 in HTSFs; a pronounced reduction in focal adhesion areas in HTSFs; decreased collagen contraction; and reduction to baseline levels of SMAA, collagen I, and FN. Our data demonstrate that Hic-5 is an essential element in the mechanism driving autocrine TGF-β1 production. Interestingly, forced expression of Hic-5 in NADFs was not sufficient to promote myofibroblast differentiation, but did increase the steady-state levels of TGF-β1 mRNA. Accordingly, these cells secreted significantly more latent-TGF-β1, but did not increase the levels of active TGF-β1 in the conditioned medium (data not shown). These data indicate that Hic-5 is directly involved in regulating latent TGF-β1 expression, whereas its role in regulating TGF-β1 activation is unknown. Taken together, our data suggest a working model in which myofibroblast differentiation is initiated by active TGF-β produced by other cell types (for example, macrophages) during the early phases of wound healing. However, active TGF-β also induces Hic-5 production, which under pathogenic conditions induces endogenous TGF-β production, which when activated, promotes an autocrine loop maintaining the pathogenic phenotype.

Hic-5 is upregulated in cells undergoing an epithelial-to-mesenchymal cell transition and is necessary for this phenotypic conversion (Tumbarello *et al.*, 2005; Tumbarello and Turner, 2007). Forced overexpression of Hic-5 in the mammary epithelial cell line, MCF10A, led to disruption of the cortically arranged actin and the development of ROCK-dependent stress fibers, indicative of a contractile mesenchymal phenotype (Tumbarello and Turner, 2007). Silencing Hic-5 mRNA with specific siRNAs suppressed TGF-β1-dependent RhoA activation during epithelial-to-mesenchymal cell transition. However, we found that in the absence of Hic-5, with addition of TGF-β1, NADFs could still differentiate into a myofibroblast and, conversely, that overexpressing Hic-5 in NADFs was not sufficient to cause fibroblast differentiation (Figure 2b and data not shown). Therefore, we hypothesize that Hic-5 is required to properly regulate a set of Rho modulators (guanine exchange factors and GTPase activating proteins) necessary for epithelial-to-mesenchymal cell transition that are distinct from those required for myofibroblast differentiation. This hypothesis has important implications for epithelial-to-mesenchymal cell transition during tumor progression and as a potential source of fibroblasts at sites of tissue injury.

Our earlier work established that an autocrine loop exists for TGF-β1 production and activation in HTSFs, and that this autocrine loop regulates the proliferation of these pathogenic myofibroblasts in a Hic-5-dependent mechanism (Dabiri *et al.*, 2006, 2008). We now report that Hic-5 is required to maintain the autocrine loop of TGF-β1 that, in turn, supports the persistent myofibroblast phenotype seen in HTSFs. Importantly, we find that the TGF-β1-dependent autocrine loop is disrupted when Hic-5 is knocked down, resulting in reversion of these myofibroblasts to a more normal fibroblast phenotype. Adding back active TGF-β1 to RNA interference-treated HTSFs restores most myofibroblast functions (Figures 1–3), but not the autocrine loop (Figure 4). Our data suggest that Hic-5 is an important new target in HTSFs, and potentially other pathogenic myofibroblasts, that could be inhibited therapeutically to help in the treatment of cutaneous fibrosis and possible other fibrotic disorders.

## MATERIALS AND METHODS

### Cell culture

All cells used in these studies were cultured (37 °C, 5% $CO_2$) in serum-containing media, which consisted of DMEM, 10% fetal bovine serum, and 1% penicillin–streptomycin (all of which were obtained from Gibco Invitrogen Corporation, Carlsbad, CA). NADFs were purchased and HTSFs were isolated as described previously (Dabiri *et al.*, 2008). HTS tissues were acquired anonymously under protocols approved by the relevant medical and ethical committees of the Shriners Burn Center in accordance with the Declaration of Helsinki Principles, and patients gave written, informed consent.

### Western blot

NADF and HTSF lysates were analyzed for protein expression by western blotting with the following primary antibodies at a concentration of 1:1000: vinculin (Sigma Aldrich, St Louis, MO), SMAA (Sigma Aldrich), FN 7.1, specific for the synergy site ($III_9$) present with FN (Neomarkers, Fremont, CA), collagen type I (Sigma Aldrich), paxillin (Sigma Aldrich), Hic-5 (BD Transduction, Franklin Lakes, NJ), and ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA). Western blots were developed as described previously (Dabiri *et al.*, 2008).

### RNA interference

NADFs or HTSFs were transfected with siRNA (Dharmacon, Lafayette, CO) using OligofectAMINE (Invitrogen, Carlsbad, CA). Hic-5 knockdown was performed initially using a pool of four

siRNAs; the pool was deconvolved and two specific siRNAs to Hic-5 were employed, duplex no. 1 (GGAGCUGGAUAGACUGAUGUU) and duplex no. 2 (GGACCAGUCUGAAGAUAAGUU). Hic-5 knockdown both with the pooled (data not shown) and the individual siRNAs (duplex no. 2, data not shown) resulted in a greater than 90% decrease in the expression of Hic-5 in both NADFs and HTSFs under basal conditions. In addition, all results using both duplexes were similar. Cells were transfected in OptiMem (Gibco Invitrogen Corporation) without serum for 4 hours. An equal volume of DMEM with serum was added and culturing was continued for 5 days. Cells were then sub-cultured for experiments as designated in the figure legends.

### Reverse transcriptase-PCR

Total RNA was extracted from cells using the PURESCRIPT RNA Isolation kit (Gentra Systems, Minneapolis, MN). RNA (one-tenth) was reverse transcribed and 1 μg of the resulting cDNA was used to detect mRNA abundance with primers for TGF-β (forward: 5′-GTACCTGAACCCGTGTTGCT, reverse: 5′-GAACCCGTTGATG TCCACTT), Hic-5 (forward 5′-GCTAGATCGGTTGCTTCAGG, reverse 5′-GCGGAAGTCAGAGAGTGAGG), and glyceraldehyde-3-phosphate dehydrogenase as control (forward: 5′-CATGGCCTC CAAGGAGTAAG, reverse: 5′-GGTTGSGCACAGGGTACTTTA). All primers were designed to give ~200–350-bp products. PCRs were carried out as previously described (Dabiri *et al.*, 2008).

### Collagen lattice

Before preparing the gels, 5 ml of BSA (Sigma Aldrich) solution (0.1% BSA in phosphate-buffered saline, filter sterilized) was pipetted into each well of a six-well plate and incubated at 37 °C for 1 hour. A 1.4-ml volume of bovine dermal collagen, Vitrogen (Cohesion, Palo Alto, CA), was mixed with 0.4 ml of 5 × DMEM in a 15-ml centrifuge tube cooled on ice. The pH was adjusted to a range of 7.2 to 7.5, using a sterile solution of 1 N NaOH. Either Hic-5 or control siRNAs were transfected into either HTSFs or NADFs. Cells were trypsinized and cultured in serum-free medium in the presence or absence of TGF-β1 (10 ng ml$^{-1}$) for 5 days. The control and modified cells were trypsinized and 0.2 ml of the cell suspension (containing $2.5 \times 10^5$ cells) was added to the collagen solution, gently mixed, and poured. Collagen lattices were allowed to gel for 60 minutes under a 5% $CO_2$ atmosphere at 37 °C. After 60 minutes, the collagen lattices were detached from the surface of the well by rimming the lattice with a sterile spatula and gently swirling the six-well plate. A 2-ml volume of serum-free medium was added to each well. Plates were incubated under a 5% $CO_2$ atmosphere at 37 °C. To measure contraction, the tissue culture dishes were placed on top of a transparent metric ruler on an opaque background, and the diameters of the lattices were recorded.

### Immunofluorescence

Cells were stained as previously described (Dabiri *et al.*, 2006), with slight modification. Control or modified cells were cultured at 30% confluence in complete medium for 18 hours, then washed with Dulbecco's phosphate-buffered saline, and serum-free medium was added, followed by addition of 10 ng ml$^{-1}$ of TGF-β1 or 20 μg ml$^{-1}$ of anti-TGF-β1 (R&D Systems, Minneapolis, MN) where specified. Cells were allowed to incubate for 5 days. On the fifth day, cells were washed fixed, permeabilized, and blocked for 1 hour. The samples were then incubated sequentially with mAbs against vinculin (1:400; Sigma Chemical Co., St Louis, MO) and then with a mixture of goat anti-mouse Alexa Fluor 488 (1:1000) and TRITC-conjugated phalloidin (1:100). Coverslips were mounted and the labeled cells were observed with an Olympus BX60 microscope equipped with an oil-immersion objective (× 100/1.3, phase 3). Images were captured with attached Cooke Sensicam digital camera and deconvolved using Slidebook 3.0.10.3 software (FFTW licensed from Massachusetts Institute of Technology, Cambridge, MA).

### Focal adhesion measurements

Focal adhesion measurements were performed as described previously (Dabiri *et al.*, 2006). In brief, cells ($n = 20$) were chosen that were not in contact with any other cell and were well spread. The results were observed using Olympus BX60 microscope equipped with an oil-immersion objective (× 60/1.25, phase 3) and images captured as described above. Vinculin staining was measured for area (μm$^2$) of focal adhesion using Image Pro-Plus version 4.5.1.26 (Media Cybermatic, Silver Spring, MD). The limits set for measurement was 1–50 μm$^2$ (6.34 pixels μm$^{-1}$).

### Measurement of TGF-β1 levels by ELISA

Conditioned serum-free media from control and modified fibroblasts were collected at the end of 5 days and the levels of TGF-β1 were measured by ELISA (R&D Systems). The antibodies used in the ELISA kit are only able to detect TGF-β1 in its active form; thus, samples were activated by acidification (HCl) before ELISA to determine the amount of latent-TGF-β1 in the conditioned media (total measured TGF-β1 − active TGF-β1 levels). After collecting the conditioned media, cells were treated with trypsin, counted, and the values of TGF-β1 were normalized by the ratio between the volume of medium in the dish and the final cell number.

### Statistics

Statistical comparisons between sample groups were made using an analysis of variance with $P \leqslant 0.005$, indicating significant difference between the groups.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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