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IL-6 Trans-signaling-STAT3 Pathway Mediates ECM and Cellular Proliferation in Fibroblasts from Hypertrophic Scar

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Abstract

The molecular mechanisms behind the pathogenesis of post-burn hypertrophic scar (HS) remain unclear. Here, we investigate the role of interleukin-6 (IL-6) trans-signaling-STAT3 pathway in HS fibroblasts (HSF) derived from burned-induced HS skin. HSF showed increased Tyr 705 STAT3 phosphorylation over normal fibroblast (NF) after IL-6•IL-6Rα stimulation by immunoassays. The endogenous STAT3 target gene, SOCS3, was upregulated in HSF and showed increased STAT3 binding on its promoter relative to NF in Chromatin Immunoprecipitation assay. We observed that the cell surface signaling transducer glycoprotein 130 is upregulated in HSF using Q-RT-PCR and flow cytometry. The production of excessive extracellular matrix (ECM), including the expression of alpha2 (1) procollagen (Col1A2) and fibronectin 1 (FN) were seen in HSFs. A STAT3 peptide inhibitor abrogated FN and Col1A2 gene expression in HSF indicating involvement of STAT3 in ECM production. The cellular proliferation markers Cyclin D1, Bcl-Xl and c-Myc were also upregulated in HSF and knockdown of STAT3 by siRNA attenuated c-Myc expression indicating the essential role of STAT3 in fibroblast proliferation. Taken together, our results suggest that the IL-6-trans-signaling-STAT3 pathway may play an integral role in HS pathogenesis and disruption of this pathway could be a potential therapeutic strategy for the treatment of burn-induced HS.

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CONFLICT OF INTERSEST

The authors state no conflict of interest.

Keywords

IL-6 trans-signaling; STAT3; ECM; Hypertrophic scar

INTRODUCTION

Exuberant fibroproliferation leading to hypertrophic scar (HS) is a common complication after thermal and other injuries for reasons that are not well understood. HS is characterized by erythematous, raised, pruritic lesion of the healing skin that significantly impairs the quality of life in affected patients (Bombaro *et al.*, 2003; Deitch *et al.*, 1983; Scott *et al.*, 2000). There is currently no definitive treatment available for HS. Although, interventions including skin grafting, pressure therapies, steroids and silicone dressings have been tried with modest success, the only effective treatment until now has been surgical removal (Sheridan and Tompkins, 2004; van Zuijlen *et al.*, 2001). One main histological characteristic of HS is the hypercellularity due to increased numbers of fibroblasts and the recruitment of inflammatory cells, producing profibrogenic factors to increase angiogenesis and abnormal ECM deposition (Castagnoli *et al.*, 1997). Thus, disruption of the balance between synthesis and degradation of ECM during the remodeling phase of wound healing may lead to abnormal scarring resulting in HS.

Recent studies evidence that the IL-6 signaling pathway, converging on Janus kinase (Jak)-signal transducer and activator of transcription (STAT)-3 complex, is important for both wound healing and in keloid pathogenesis (Ghazizadeh *et al.*, 2007; Lim *et al.*, 2006). IL-6 is a multifunctional cytokine that is upregulated in response to inflammation, oxidative stress, vascular damage, and burn injuries (Hou *et al.*, 2008b; Finnerty *et al.*, 2006). IL-6 production by inflammatory cells and dermal fibroblasts has been linked to the pathogenesis of fibrosis associated with rheumatoid arthritis, systemic sclerosis, pulmonary interstitial fibrosis and psoriatic lesions (Tan *et al.*, 1990; Shahar *et al.*, 1996; Feghali *et al.*, 1994; Sano *et al.*, 2008).

IL-6 signals to responsive cells via a “classic” signaling pathway by binding cell-surface IL-6Rα, or by “trans-signaling,” mediated by IL-6 and soluble IL-6Rα (sIL-6Rα) binding cell-surface glycoprotein 130 (gp130). In the classic signaling pathway, binding of IL-6 to IL-6Rα triggers formation of a membrane-bound hexameric IL-6•IL-Rα•gp130 high-affinity complex (Scheller and Rose-John, 2006; Jones *et al.*, 2001). Receptor ligation induces conformational changes in the cytoplasmic domains of gp130 that bring JAKs into close proximity. Phosphorylation of gp130 by JAKs then activates STATs, particularly isoforms -1 and -3. Activated STAT3 rapidly translocates into the nucleus, where it binds enhancer sequences of genes that control fundamental cellular processes, including acute phase response, survival, proliferation, differentiation, and wound healing (Kushner, 1988; Dauer *et al.*, 2005; Yamauchi-Takihara and Kishimoto, 2000).

In contrast, the IL-6 trans-signaling pathway is mediated by formation of a soluble IL-6•sIL-6Rα complex that binds gp130 and activate STAT3 in any cell type, extending the actions of IL-6 to cells that do not normally express IL-6Rα (Jones *et al.*, 2005; Vermes *et al.*, 2002). The release of sIL-6Rα is known to occur either by differential mRNA splicing

or by proteolytic cleavage mediated by activated leukocytes, (Ebihara *et al.*, 2011). Remarkably, recent studies revealed that the IL-6•sIL-6Rα complex is induced in chronic inflammatory diseases, in particular Crohn's disease, rheumatoid arthritis, and inflammatory colon cancer where it may be important in disease maintenance (Jones *et al.*, 2001).

Although studies of the human burn response show induction of IL-6 secretion and acute-phase reactant synthesis (Jeschke *et al.*, 2008), the role of IL-6 trans-signaling and activation of the gp130-STAT3 pathway in mediating exaggerated scar formation have not been explored. In this paper, we hypothesized a role for the IL-6 trans-signaling pathway in HS formation and showed that compared to NF, HSFs show an exaggerated STAT3 response to IL6•sIL-6Rα and phosphorylated STAT3 caused induction of membrane bound gp130 expression. Finally, we show that excessive ECM synthesis and cellular proliferation are suppressed by a cell permeable STAT3 peptide inhibitor and STAT3 siRNA respectively. Thus, these data reveal an indispensable role of STAT3 downstream of the IL-6 trans-signaling pathway in HS pathogenesis and STAT3 inhibition may serve as a therapeutic target for the treatment of HS fibrosis.

RESULTS

Expression of phosphorylated STAT3 in postburn HS

To determine whether STAT3 participates in HS formation, paraffin-embedded HS sections from recovering burn patients were used to perform IHC analysis with anti-phospho-Tyr705 STAT3 antibody (Ab) or with control rabbit IgG. In Figure 1a, panel A-C (10X) and D-F (20X), activation of phospho-STAT3 was confirmed by the presence of positive nuclear staining for phospho-Tyr705 STAT3 in the dermal fibroblast nuclei of burned skin with HS. Staining of HS skin with rabbit IgG (panel G) and normal skin with phospho-Tyr705 STAT3 Ab (panel H-I) did not show any significant nuclear staining in the dermis, indicating specificity of the phopho-Tyr705 STAT3 Ab and upregulation of phospho-STAT3 in HS. Increased dermal staining intensity was measured in HS and is shown in Figure 1b.

Next, we determined the induction of activated STAT3 in primary HS fibroblast (HSF) derived from burned patient with severe HS and in normal fibroblast (NF) derived from the non-burned area of the same patient. We first confirmed the presence of fibroblast cells in the post-burn HSF by staining with fibroblast marker α-smooth muscle actin (α-SMA) Ab and also measuring α-SMA gene expression by RT-PCR. The supporting data (Figure S1) show a high level of immunoflurescence staining in HSF with α-SMA Ab and upregulated α-SMA gene expression in HSF compared to NF (Figure S2). Next, we performed Western immunoblot analysis of whole cell extract (WCE) isolated from HSF and NF with phopho-Tyr705 STAT3 Ab. In Figure 1c, upper panel, we observed that in contrast to NF, HSFs showed a 2-fold increase in phospho-STAT3 signal, indicating STAT3 activation occurs in HS. The lower panel shows β - actin expression as loading control. Activation of phospho-STAT3 by Western blot by other HSFs are presented in supporting Figure S3.

Activation of STAT3 target gene in HSF by IL-6 trans-signaling pathway

Because normal fibroblast cells do not express membrane bound IL-6Rα, we hypothesized that STAT3 could be activated in HS via the IL-6 trans-signaling pathway. Here we treated the NF and HSF cells with IL-6•sIL-6Rα or left untreated and measured expression of the STAT3-regulated human suppressor of cytokine signaling (hSOCS3) gene in total RNA by Q-RT-PCR. In contrast to NFs, hSOCS3 expression in HSFs was induced both under basal conditions and in response to IL-6•sIL-6Rα (Figure 2a), indicating that HSFs have enhanced sensitivity to IL-6 trans-signaling. We also measured the kinetics of activated STAT3 expression in HSF and NF cells in the presence of IL-6•sIL-6Rα by quantitative bioplex phosphoprotein detection assay. We observed that phospho-Tyr705 STAT3 expression is higher in HSF than in NF and peaked (~ 2.5 fold) at 30 min after IL-6•sIL-6Rα stimulation which gradually decreased after 60 min (data not shown). To prove the involvement of STAT3 in HSF sensitivity to IL-6 trans-signaling, the effect of reducing endogenous STAT3 level was investigated. For this purpose, RNA interference was used to reduce STAT3 expression in HSFs and NFs, where activated STAT3 levels were reduced by more than 70% with STAT3 siRNA as compared to cells transfected with non-target control siRNA, Figure 2b. In Figure 2c, hSOCS3 gene expression was attenuated in the presence of STAT3 siRNA indicating the role of STAT3 in target gene activation during HS formation.

Enhanced STAT3 recruitment to the hSOCS3 promoter in HSF

To establish that enhanced sensitivity of HSF to IL-6 trans-signaling is due to increased STAT3 recruitment on its target hSOCS*3* promoter, we utilized a two step chromatin immunoprecipitation (ChIP) assay on chromatin isolated from IL-6•sIL-6Rα stimulated NFs and HSFs. Figure 3 shows that in the presence of IL-6•sIL-6Rα the promoter abundance of STAT3 is significantly increased in HSF compared to that in NF. This result indicates that increased STAT3 occupancy in HSF is required for enhanced target gene expression and sensitivity to IL-6-trans-signaling.

Increased expression of cell surface gp130 in HSF

Gp130 is the upstream rate limiting signal transducer of the IL-6•sIL-6Rα complex. To evaluate the possibility that the increased trans-signaling seen in HSFs is due to upregulation of the gp130 (O'Brien and Manolagas, 1997), gp130 mRNA was measured by Q-RT-PCR in IL-6•sIL-6Rα stimulated NF and HSF cells. We observed increased levels of gp130 transcript (~3 fold) in HSF compared to NF, explaining the exaggerated response of HSF cells to IL-6•sIL-6Rα (Figure 4a). We further analyzed the cell surface expression of gp130 by flow cytometry in IL-6•sIL-6Rα stimulated HSF and NF cells. In Figure 4b flow cytometry data showed increase in mean fluorescence intensity of gp130-phycoerythrin (PE) in HSF cells from 965 to 1692 arbitrary units when stimulated with IL-6•sIL-6Rα. Further, there are significantly higher amounts of gp130-expressing HSF cells (approximately ~ 60%) than NF cells under stimulated conditions. These exciting findings suggest that HSFs have upregulated gp130 expression and thereby show enhanced sensitivity to the IL-6 trans-signaling pathway.

STAT3 inhibitor abrogates production of ECM in HSF

To test that high STAT3 activation in burn-induced HS upregulates ECM gene expression, we examined ECM production in HSFs and NFs. We measured the mRNA expression of alpha2 (I) procollagen (Col1A2) and fibronectin 1 (FN), two principal ECM molecules involved in keloid formation (Ghazizadeh *et al.*, 2007). Total cellular RNA was isolated, and expression of the STAT3-regulated human Col1A2 and FN gene was measured by Q-RT-PCR. In Figure 5a, contrast to NFs, expression of Col1A2 and FN gene was induced in HSFs, indicating increased ECM production. To investigate the functional role of STAT3 in ECM synthesis we used a cell permeable STAT3 peptide inhibitor previously reported to inhibit STAT3 but not STAT1 (Timofeeva *et al.*, 2007). For this purpose we first determined the effect of this peptide on hSOCS3 expression. We treated NFs and HSFs with the STAT3 inhibitor peptide (500 nM) for 6 hours and stimulated with IL-6•sIL-6Rα for 30 minutes before harvest. Total RNA was then subjected to Q-RT-PCR for the hSOCS3 gene. As seen in Figure 5b, the inhibitor peptide had very little or no effect on NFs, whereas in HSFs, induction of the hSOCS3 gene was abrogated in the presence of the peptide, indicating a role of activated STAT3 in HSFs. We next examined the effect of this STAT3 inhibitor on FN and Col1A2 gene expression. In contrast to NF, inhibitor treatment of HSFs abrogated the expression of IL-6•sIL-6Rα-induced FN (Figure 5c) and Col1A2 (Figure 5d) gene expression, indicating the requirement for STAT3 in ECM production in burn-induced HS.

Requirement for STAT3 in increased proliferation of HSFs

STAT3's role in fibroblast proliferation following a burn injury is incompletely understood. Previous work has shown that fibroblasts from burned patients with keloids have increased growth rates compared to non-lesion fibroblasts (Ghazizadeh *et al.*, 2007). As we also observed a similar higher growth rate in post-burned HSF than NF (Figure S4), here we measured the expression of cellular proliferation marker Cyclin D1, c-Myc, and Bcl-xl in HSFs and NFs. As expected, mRNA expression of Cyclin D1, c-Myc, Bcl-xL gene were upregulated in HSFs as compared to NFs indicating exaggerated fibroblast proliferation associated with scar formation (Figure 6a). To assess the direct effect of STAT3 in HSF proliferation, cells were transfected with STAT3 siRNA or non-target control siRNA and total RNA was extracted for c-Myc expression. In Figure 6b, a significant decrease in c-Myc expression was observed in NF and HSF cells in the presence of STAT3 siRNA indicating the role of STAT3 activation in ECM synthesis and cellular proliferation, contributing to HS pathogenesis.

DISCUSSION

The systemic inflammatory response after burn injury releases large quantities of proinflammatory cytokines, including IL-6 (Finnerty *et al.*, 2008). In macrophage-inflamed tissues, a soluble IL-6 receptor (sIL-6Rα) is formed, that when bound to IL-6 acts as an agonist on dermal fibroblasts that express the cell-surface signal transducer gp130. Binding of IL-6•sIL-6Rα to gp130 activates the transcription factor STAT3 via the IL-6 trans-signaling pathway, a pathway known to be crucial in the pathogenesis of inflammatory diseases (Jones *et al.*, 2005). The pathophysiology of HS, characterized by fibroblast proliferation and excessive collagen deposition, remains largely unknown. Although few

studies have shown that cytokines, in particular, IL-6, plays an important role in the pathogenesis of keloid scar (Ghazizadeh *et al.*, 2007; Lim *et al.*, 2006), activation of the IL-6 trans-signaling-gp130-STAT3 pathway and its downstream functional roles in the pathogenesis of HS has not been documented. In this study, we have established the activation of gp130-STAT3 signaling via a IL6-trans-signaling pathway in HS fibroblasts. Our data show that activation of STAT3 leads to upregulation of STAT3 target genes including those controlling ECM production and cellular proliferation and may also regulate the upstream signal transducer gp130. Activation of these genes by increased STAT3 occupancy in their promoter leads to abnormal wound healing, excessive collagen deposition and fibroproliferative growth in HS lesions.

The key element of this study is the transcription factor STAT3 which we showed by IHC being activated by tyrosine phosphorylation in human HS tissue sections. Dermal fibroblasts are the main regulatory cells which are known to increase in HS lesions by secreting collagen, cytokines and chemokines. These secreted molecules induce fibroblast proliferation and migration of inflammatory cells leading to fibrosis (Sidgwick and Bayat, 2012). Although dermal fibroblasts cells are known to produce a large amount of IL-6, several studies have shown that they do not express enough membrane bound IL-6Rα and are thought to respond to IL-6 mainly through the IL-6 trans-signaling pathway (Sporri *et al.*, 1999; Jones *et al.*, 2005). This information led us to test our hypothesis that activation of STAT3 in HS occurs via the IL-6 trans-signaling pathway. Our observation that addition of IL6•sIL-6Rα stimulated STAT3 phosphorylation in primary HSF and concomitant activation of STAT3 target gene *in vitro* support our hypothesis that activation of STAT3 in HS may also act *in vivo* by IL-6 trans-signaling pathway.

Formation of the IL-6•sIL-6Rα complex initiates transduction through the membrane bound gp130 signal transducer leading to the activation of JAK-STAT3 signaling pathways. Gp130 is a shared receptor for the IL-6 cytokine family and so modulation of gp130 synthesis by its ligands may serve to replenish receptor consumption. Our Q-RT-PCR and FACS analysis showed upregulation of gp130 expression and increased cell surface gp130 in HSFs explaining the exaggerated response of HSF to stimulation by the IL-6•sIL-6Rα. IL-6•sIL-6Rα has not been previously shown to regulate gp130 or gp130 post-translational processing. However, (O'Brien and Manolagas, 1997) a previous study confirmed the presence of a STAT3 response element in the gp130 promoter. Thus, consistent with this study on HSF, robust activation of STAT3 by IL-6 trans-signaling promotes increased gp130 transcription and protein expression and therefore IL-6•sIL-6Rα-STAT3-gp130 may act in a positive feedback loop.

STAT3 is a transcription factor that affects promoter activity of its target gene by binding to a specific cis-element (Ray *et al.*, 2010). Using ChIP assay, we provide direct evidence that increased binding of STAT3 to hSOCS3 promoter in HSF leads to enhanced hSOCS3 expression. We previously showed that STAT3 is acetylated as a direct target of the p300 coactivator *in vivo* and *in vitro* (Eckner *et al.*, 1994; Ray *et al.*, 2005) and this modification induces STAT3 binding to CDK-9, a member of the positive transcription elongation factor-b (PTEF-b) (Hou *et al.*, 2007; Hou *et al.*, 2008a).The role of STAT3 acetylation and recruitment of PTEF-b, in understanding gene regulation in HSFs will be studied in future.

The ultimate fate of HS is the exuberant synthesis, deposition and remodeling of the ECM by fibroblast cells, leading to fibrosis. HSFs and Keloid fibroblasts are known to produce excessively many types of ECM, such as Collagen type I and VI, and fibronectin (Sidgwick and Bayat, 2012; Abergel *et al.*, 1985). We report here that STAT3 plays a major role in the excessive collagen deposition by fibroblasts, leading to HS. Inhibition of STAT3 by a cell permeable STAT3 inhibitor showed a corresponding decline in collagen and fibronectin expression, suggesting that ECM production may be regulated by STAT3 at the transcription level.

Fibroproliferation is the characteristic of HS and Keloids that are known to involve the overgrowth of fibroblast (Ghazizadeh *et al.*, 2007; Lim *et al.*, 2009; Matsui *et al.*, 2011). Although a large body of work has shown that STAT3 promotes proliferation via its role as a transcription factor, inducing expression of oncogenes, cell cycle regulatory genes and anti-apoptotic genes (Haga *et al.*, 2003; Sinibaldi *et al.*, 2000; Bowman *et al.*, 2001), IL-6-STAT3 pathway may not be the only pathway controlling this process. Tao *et al.*, 2010 has observed involvement of JAK-STAT1 pathway in proliferation and differentiation of HSF by CTGF which is a downstream mediator of TGFβ1 and has been widely implicated in wound healing, scarring and tissue fibrosis (Igarashi *et al*.,1996; Penn *et al*., 2012; Zunwen *et al*., 2012). Here, our observation of enhanced expression of proliferation marker Cycline D1, c-Myc and Bcl-XL in HSFs and attenuation of cellular proliferation genes by STAT3 siRNA, showed a direct role for STAT3 in the fibro-proliferation of HS.

In summary, we have shown that STAT3 is activated in HS by IL-6 trans-signaling pathways that mediate ECM production and dermal fibroblast proliferation. These results elucidate the signal transduction mechanism by STAT3 in HS, and therefore may aid in the development of STAT3 antagonists as translational therapies for burn-induced scarring. The development of a nontoxic, topical therapy to decrease HS would significantly benefit the one million people burned annually, by negating the need for repeated surgeries and thereby improving the long term quality of life.

MATERIALS AND METHODS

Cell Culture and Tissue Biopsies

Skin and scar biopsies were acquired during scar revision surgeries performed between 6 and 21 months post-burn. Samples were immediately stored in formalin for paraffin embedding or in 15 % fetal calf serum in DMEM with 1% penicillin-streptomycin sulfate-amphotericin B for fibroblast isolation as described previously (Zhang *et al.*, 2011). Patients enrolled in the study were between 0 and 18 years of age at the time of injury with at least 30% of the total body surface area burned, and requiring at least one operative intervention. This study was part of a large clinical trial (www.clinicaltrials.gov, NCT00675714) evaluating the outcomes of burn survivors. The study protocol and informed consent were in compliance with Declaration of Helsinki Principles and was approved by the Institutional Review Board of The University of Texas Medical Branch (Galveston, TX). In this study we have used ten HS-tissue biopsies for IHC and all experiments with primary fibroblasts (HSF and NF) were conducted on three patient samples at passages 3 to 7.

Reagents and Antibodies

Sources of primary antibody (Ab) for ChIP assay and Western Blot included Santa Cruz Biotechnology anti-STAT3 C20 and anti-phospho-Tyr STAT3 B7 Ab respectively. For IHC we used Phospho-STAT3 (Tyr 705)(D3A7)XP mAb from New England Bio Labs. Recombinant human IL-6 and sIL-6Rα were obtained from Pepro Tech Inc. (Rocky Hill, NJ). Cell permeable STAT3 peptide inhibitor (STAT3-Hel2a-Penetratin) was synthesized at UTMB Protein Chemistry laboratory.

Western Blot Analysis

WCEs were prepared by lysing NFs and HSFs in modified RIPA buffer and fractionated by 10% SDS-PAGE as previously described (Ray *et al.*, 2008).

Immunohistochemistry (IHC)

Activated STAT3 was detected on paraffin embedded HS tissues using Phospho-STAT3 (Tyr 705)(D3A7)XP Rabbit mAb from New England Bio Labs following the manufacturer's protocol.

Bio-Plex Phosphoprotein Detection

Phospho-Tyr705 STAT3 was detected in NFs and HSFs with a Bio-Rad phosphoprotein immunoassay kit using the Bio-Plex 100 sysytem, according to the manufacture's protocol. Data analysis were done using Bio-Plex Manager software version 5.

siRNA transfection

STAT3 siRNA (Dharmacon) were transfected in NFs and HSFs (70% confluent) by TransIT-siQUEST transfection reagent (Mirus, Madison, WI) at 75 nmol/L final concentration, following manufacturer's instructions. 72 h after transfection cells were stimulated in serum free media and harvested for protein or total RNA extraction.

Quantitative-Real Time-PCR (Q-RT-PCR)

Total cellular RNA after extraction with Tri Reagent (Sigma), was subjected to reverse transcription using iScript cDNA Kit (Bio Rad). cDNA products was amplified in 20μl reaction containing 10μl iQ SYBR Green Super Mix (Bio Rad) and 400 nM primer as previously described (Ray *et al.*, 2010). To normalize template input, GAPDH transcript level was measured for each sample. Data are expressed as fold change after normalizing to GAPDH. The human primers used for RT-PCR are as follows: FN, sense primer (SP): 5'GCGCCGGCTGTGCTGCACAGG-3' and anti-sense primer (AS): 5'GCCTGGGGACAGCGGTGCCC-3'; Col1A2, SP: 5'CAGCCCCGTGGCCACGTCCC-3' and AS: 5'CCACAGGCCCTCCTGGTCCA-3'; Cyclin D1, SP: 5'GTGCTGCGAAGTGGAAACC-3' and AS: 5'ATCCAGGTGGCGACGATCT-3'; c-Myc, SP: 5'ATTCTCTGCTCTCCTCGA-3' and AS: 5'TCTTGGCAGCAGGATAGT-3'; Bcl-xl, SP: 5'GTTGAAGCGTTCCTGGCCCTTT-3' and AS: 5'CAGAATGGACTGAATCGGAGAT-3'; SOCS3, SP: 5'CAAGGACGGAGACTTCGATT-3' and AS: 5' GACTGGGTCTTGACGCTGA-3' and

Gp130, SP:5‘CTGTATCACAGACTGGCAACAAG-3‘ and AS: 5’GCATTTGCTCTCTGCTAAGTTCC-3‘

Flow Cytometry

Cultured NFs and HSFs, after IL-6•sIL-6Rα stimulation, were harvested with 1mM EDTA in PBS and washed with FACS buffer as previously described (Tieu *et al.*, 2009). Cells were Fc-blocked using purified human IgG and then either incubated with fluorescence-conjugated anti-human gp130-PE antibody (R&D systems, FAB228P) or corresponding isotype control Ab (R&D systems, IC002P) according to the manufactures’s instruction. Samples were analyzed by FACS Canto (BD) and data was analyzed with FlowJo software.

Chromatin Immunoprecipitation (ChIP) Assay

Two-step ChIP was performed with 4–6 $\times 10^6$ NF and HSF cells, after IL-6•sIL-6Rα stimulation as previously described (Nowak *et al.*, 2005). The promoter specific primers used for Q-gPCR for hSOCS3 are, SP: 5’AGCCTTTCTCTGCTGCGAGT-3’ and AS: 5’ AGCAGGGAGTCCAAGTCG-3’.

Statistical analysis

Experiments were carried out in triplicates and the data are expressed as mean±SD. Statistical analysis was performed using two-tailed student t-test, with significance set at $p<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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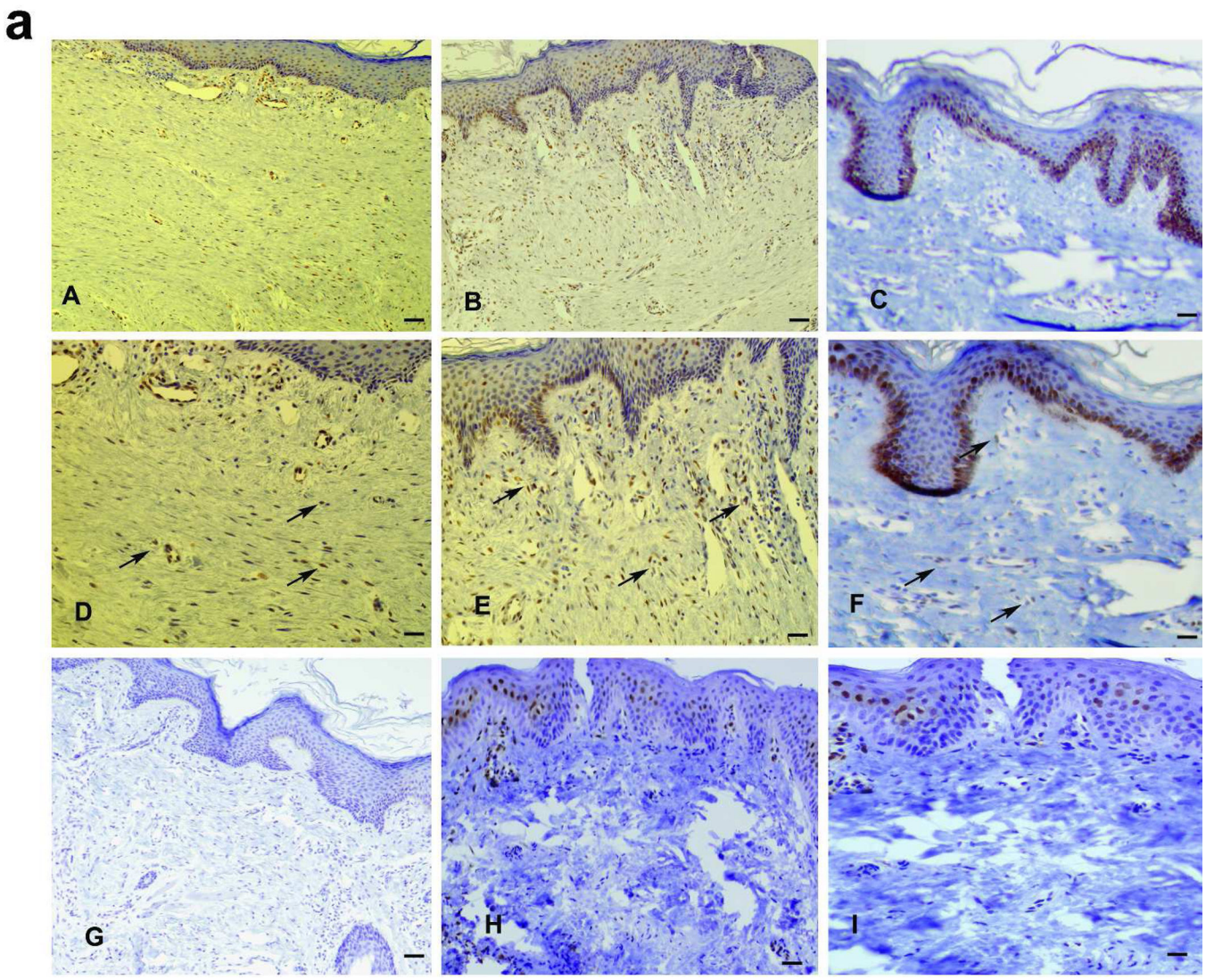
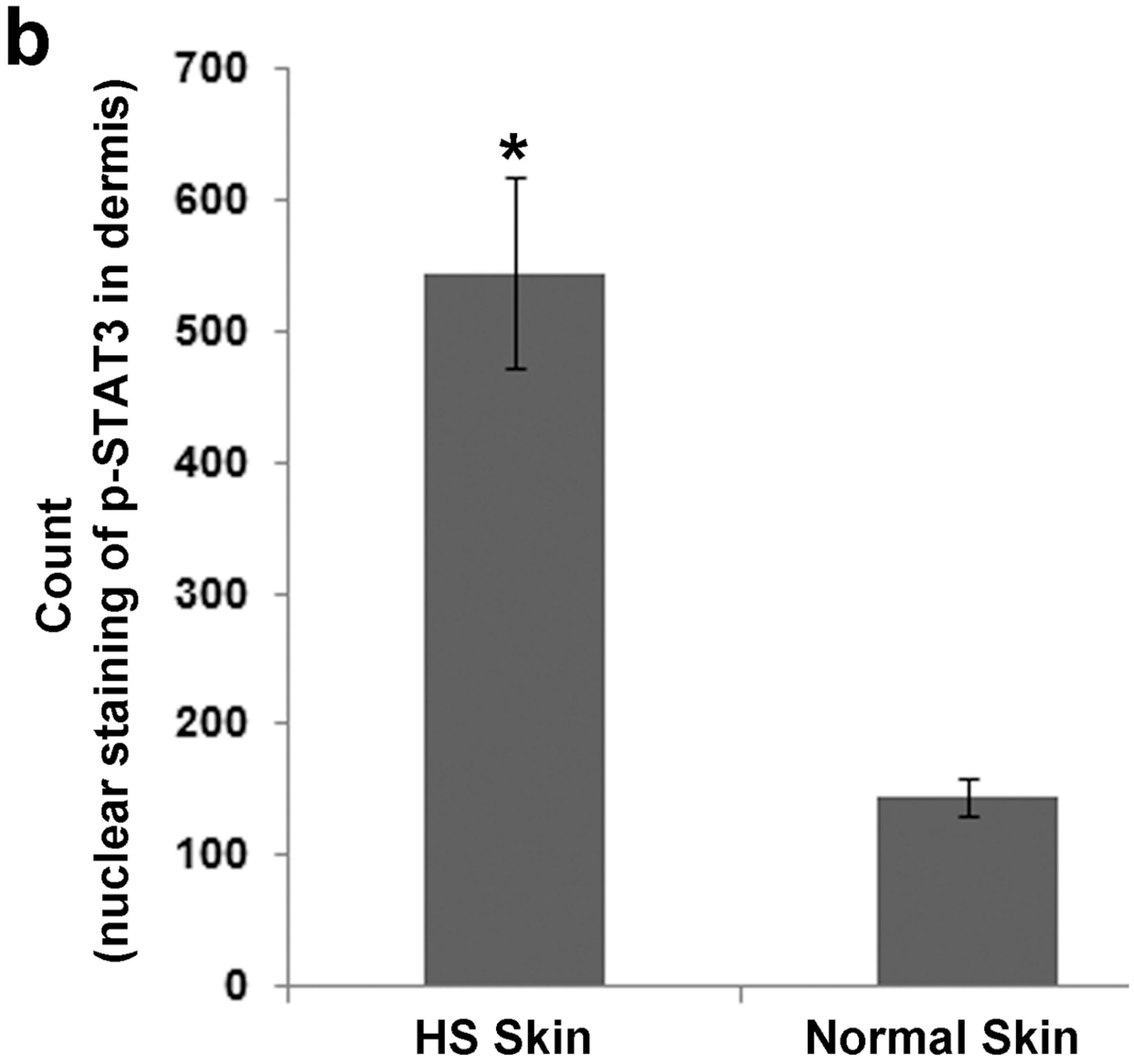




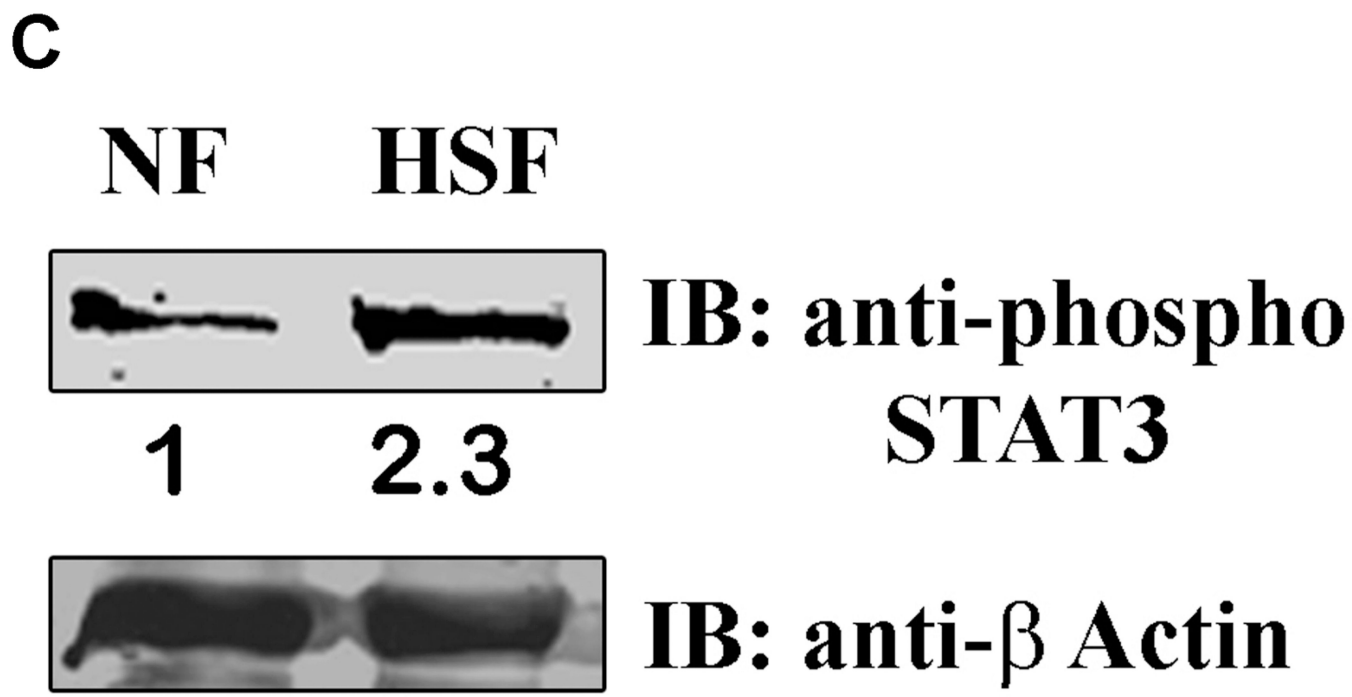
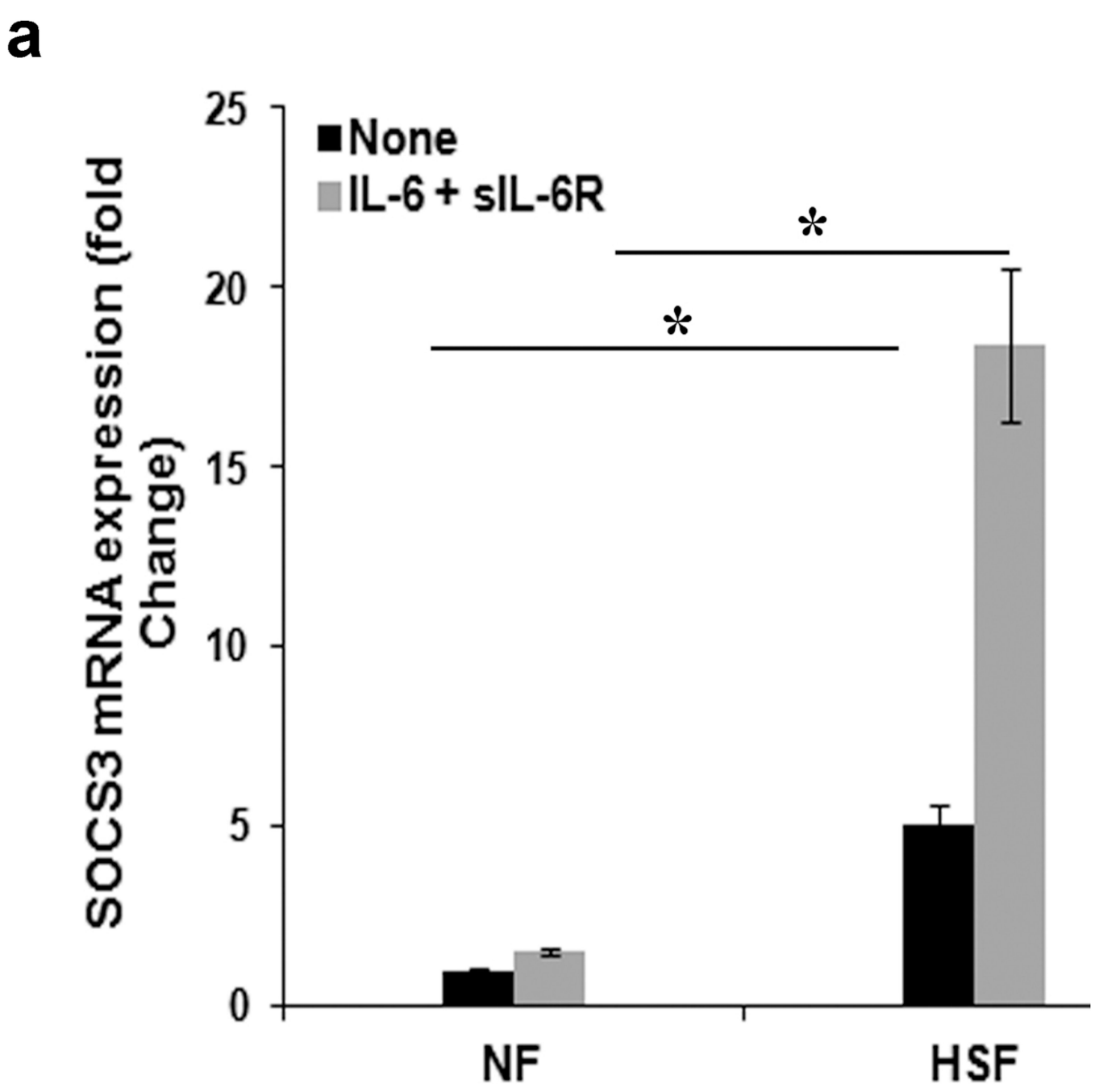


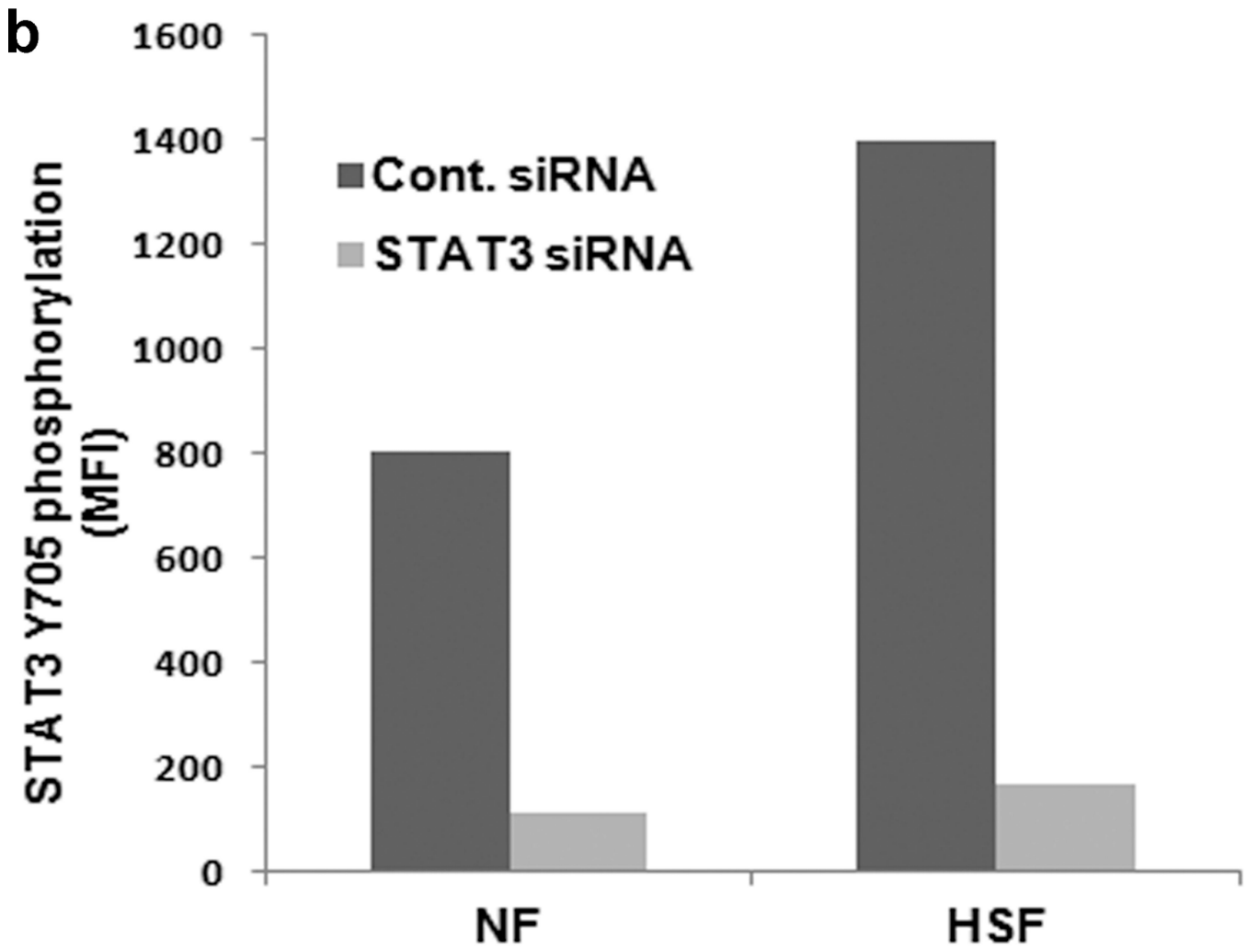
Figure 1.

(a) Immunohistochemical analysis of phospho-STAT3 (Tyr 705) expression in burned HSskin. 'A-F' and G are HS skin stained with anti-phosphoTyr705-STAT3 Ab and control IgG respectively. Panel H-I are normal skin stained with anti-phosphoTyr705-STAT3 Ab. Arrowheads (D-F) show the strong nuclear staining for activated STAT3. A-C, G-H and D-F, I are X10 and X20 magnifications respectively, *scale bar*: 10μm.

(b) Intensity of nuclear staining in the dermis of HS (n=10) and normal skin (n=5) (MetaMorph 7.3). *, $p<0.01$.

(c) Western Immunoblot analysis of phospho-STAT3 (Tyr 705) in HSFs. WCE of NF and HSF were fractionated and Western immunoblot analysis with phospho-STAT3 (Tyr 705) Ab is shown. The numbers at the bottom of the lane indicate the fold induction. β-actin blot (lower panel) shows loading control.





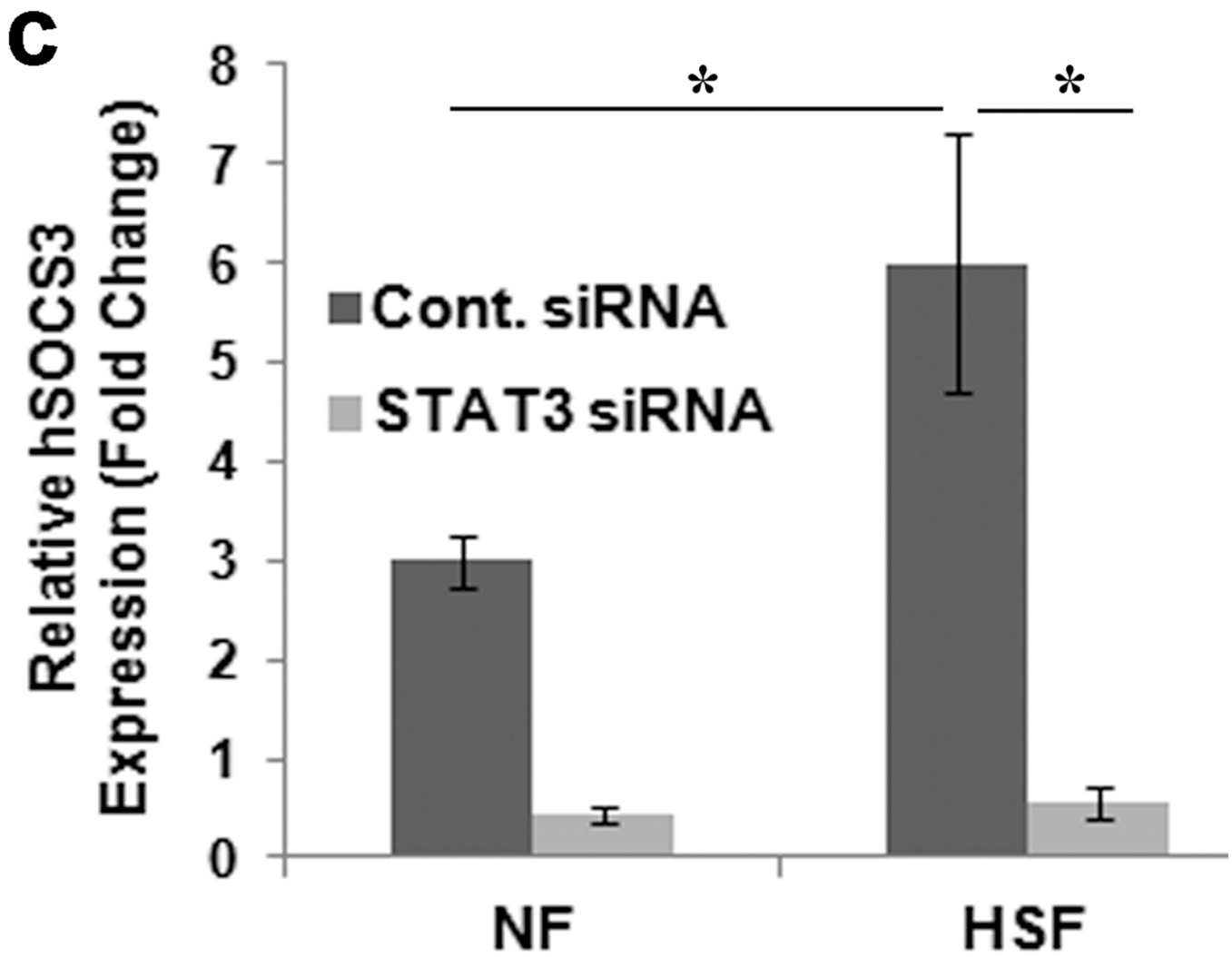


Figure 2.

(a) Activation of IL-6 trans-signaling in HSF. NF and HSF cells were treated with IL-6•sIL-6Rα (8ng/ml •25ng/ml) for 30 minutes, or left untreated. Total cellular RNA was subjected to Q-RT-PCR for hSOCS3 transcript. Shown is fold change mRNA expression relative to GAPDH. Data represents mean±SD, *, $p<0.01$.

(b) Inhibition of phospho-STAT3 with STAT3 siRNA. NFs and HSFs were transfected with either control or STAT3 siRNA. 72 hours after transfection, cells were stimulated as above and WCE were measured for phosphoTry705-STAT3 by immunoassay (Bio-Rad). Shown is the representative fluorescence intensity of phospho-STAT3 relative to total protein.

(c) Inhibition of hSOCS3 gene with STAT3 silencing. Cells were treated as in **(b)** and total cellular RNA was subjected to Q-RT-PCR for hSOCS3 expression relative to GAPDH. *, $p<0.05$.

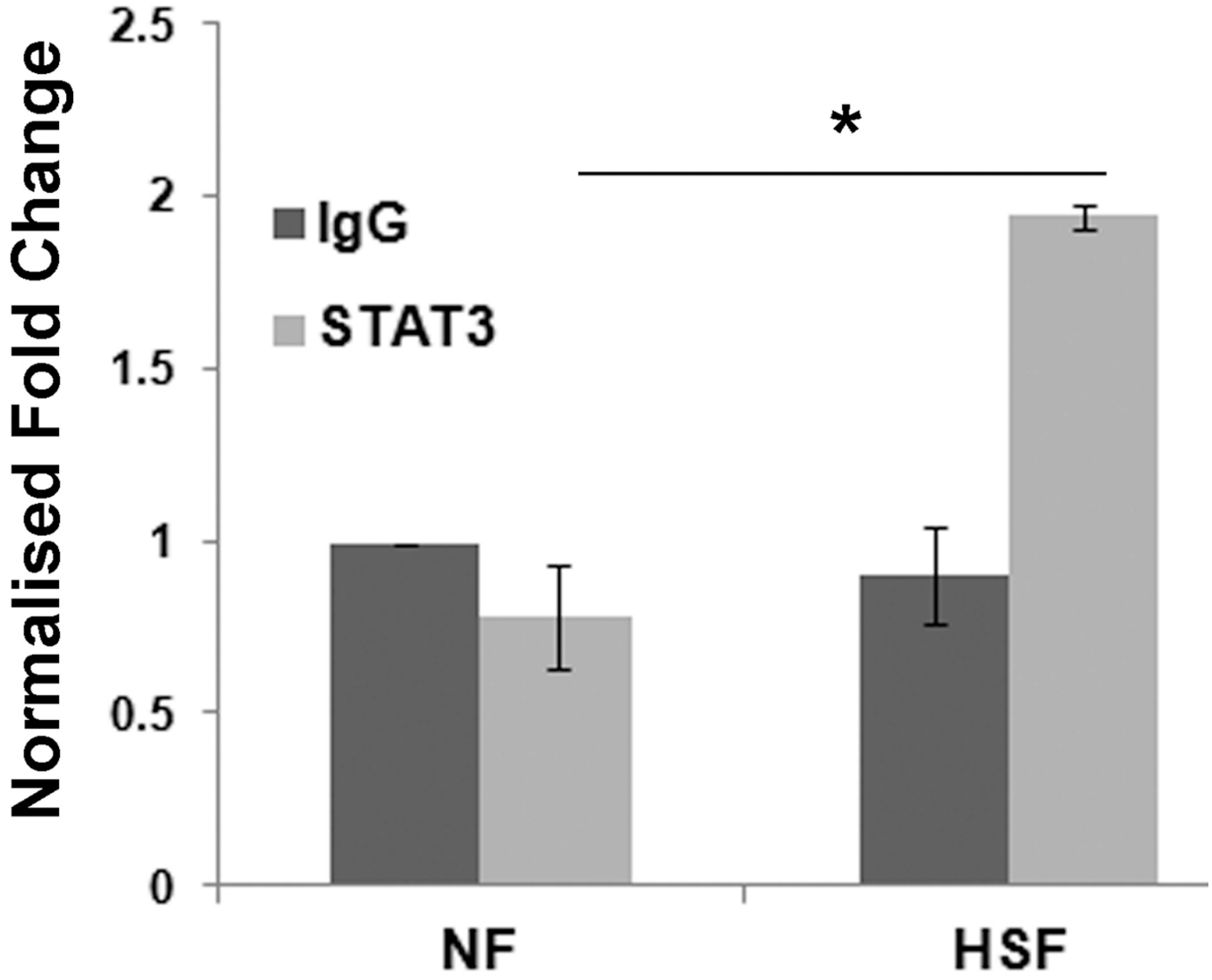
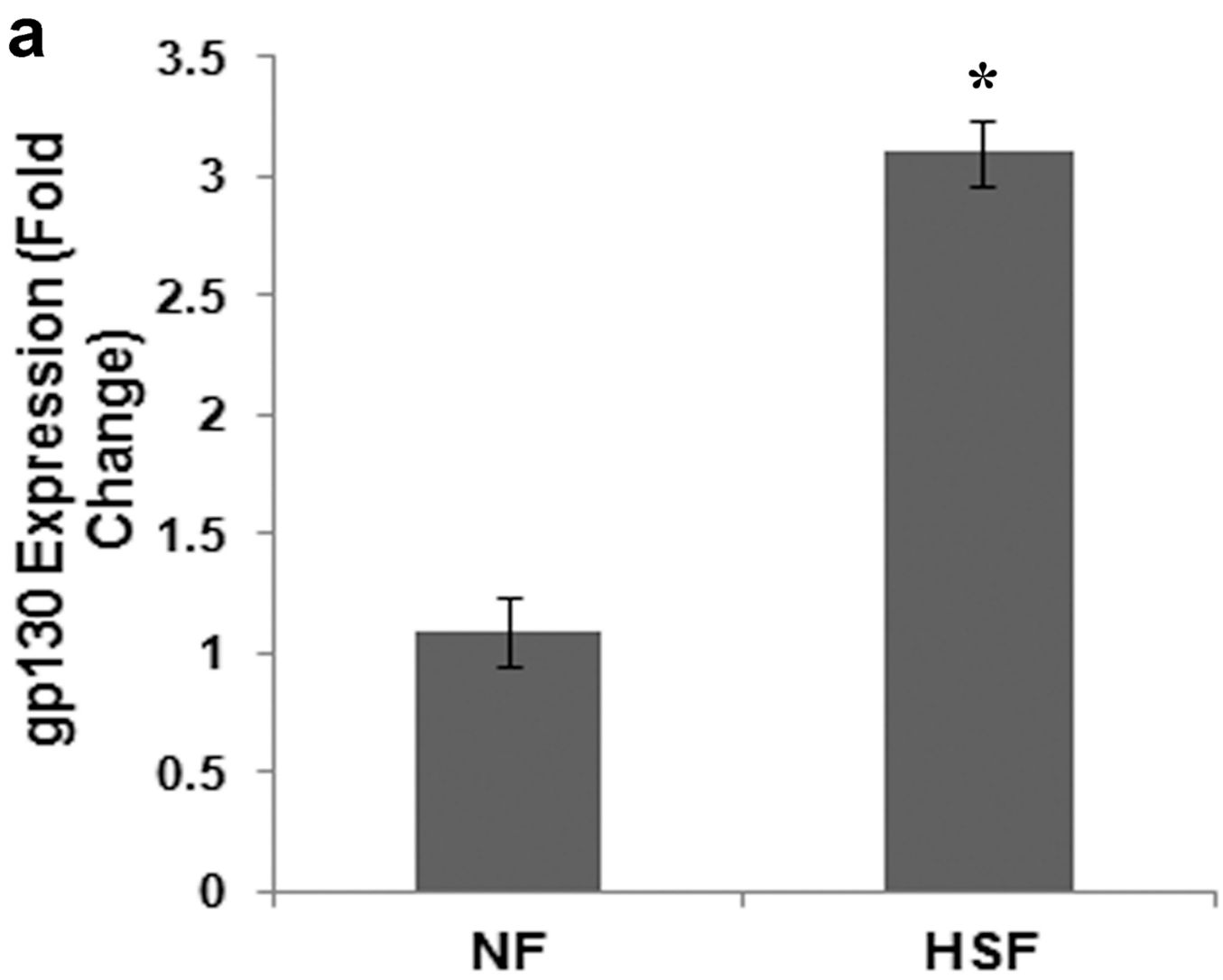


Figure 3.

hSOCS3 promoter occupancy of STAT3 in HSF and NF. Protein-DNA crosslinked extracts of IL-6•sIL-6Rα (8ng/ml •25ng/ml) stimulated NF and HSF cells were immunoprecipitated with IgG or anti-STAT3 Ab. SOCS3 promoter occupancy of STAT3 were detected by two step ChIP assays as described in the method. Shown is the fold change in quantitative-genomic PCR (Q-gPCR) normalized to input DNA. *, $p<0.01$, students t-test.



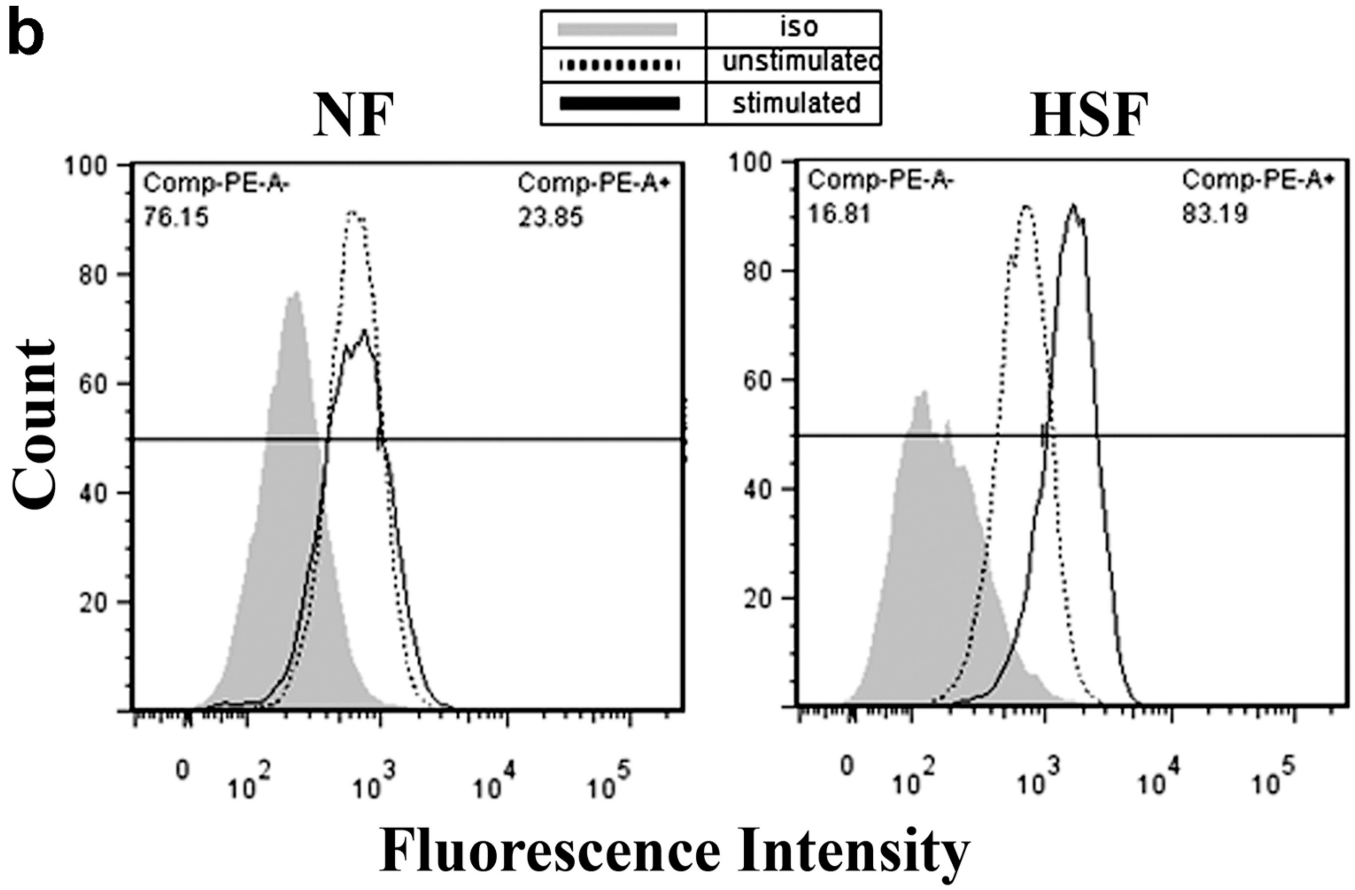
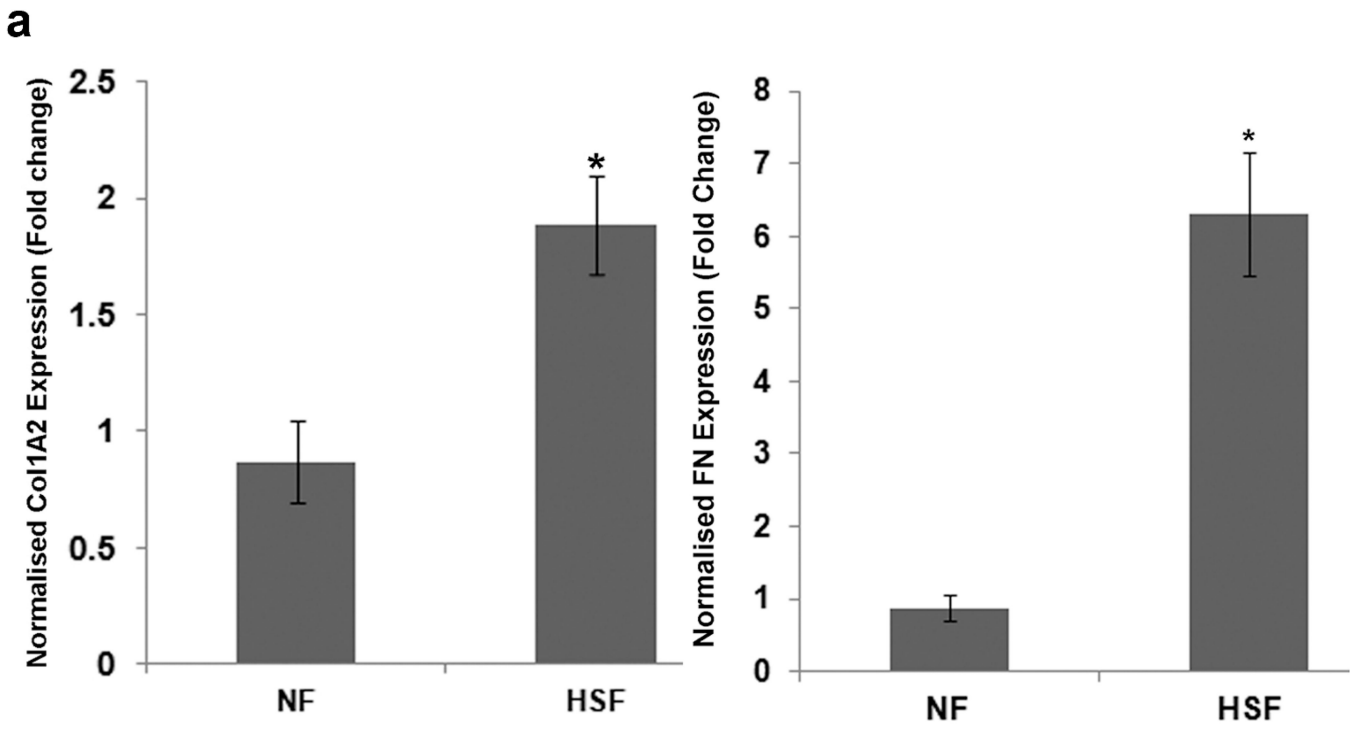


Figure 4.

(a) Activation of gp130 in HSF. NFs and HSFs were treated with IL-6•sIL-6Rα for 30 min. Total RNA was subjected to Q-RT-PCR for human gp130 mRNA expression, carried out in triplicate. Shown is fold change mRNA expression relative to GAPDH as internal control. Data represents mean±SD *, $p<0.01$, students t test.

(b) Cell surface activation of gp130 in HSF. Cultured NF and HSF cells were left untreated or IL-6•sIL-6Rα (8ng/ml •25ng/ml) stimulated for 30 min. The expression of cell surface gp130 was analyzed by flow cytometry after staining with anti-gp130-PE. Events were plotted as a function of fluorescence intensity (x-axis). Shaded histograms represent isotype antibody control and open histograms represents either unstimulated anti-gp130-PE stained cells (dotted line) or IL-6•sIL-6Rα stimulated anti-gp130-PE (solid line) as indicated.



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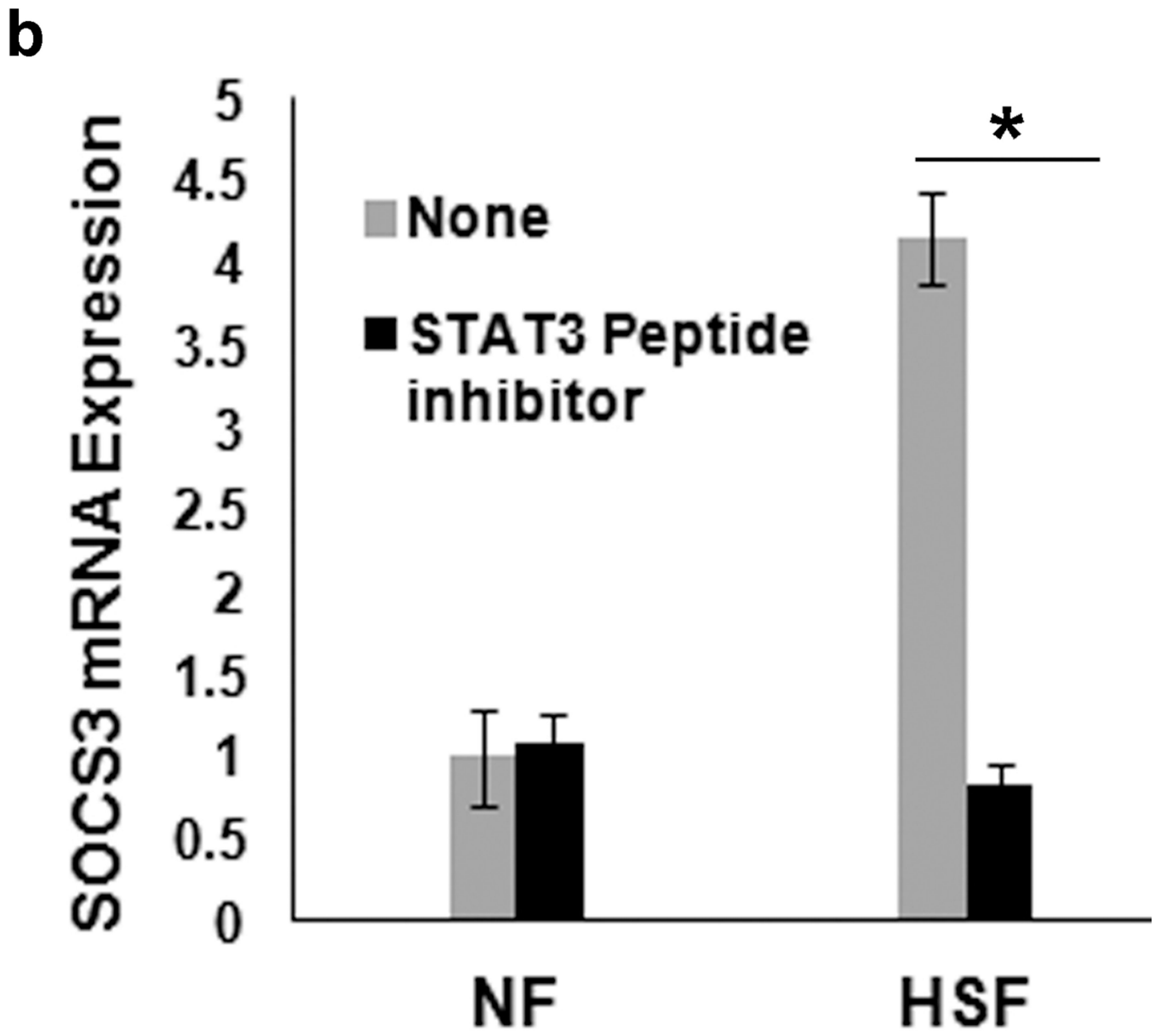
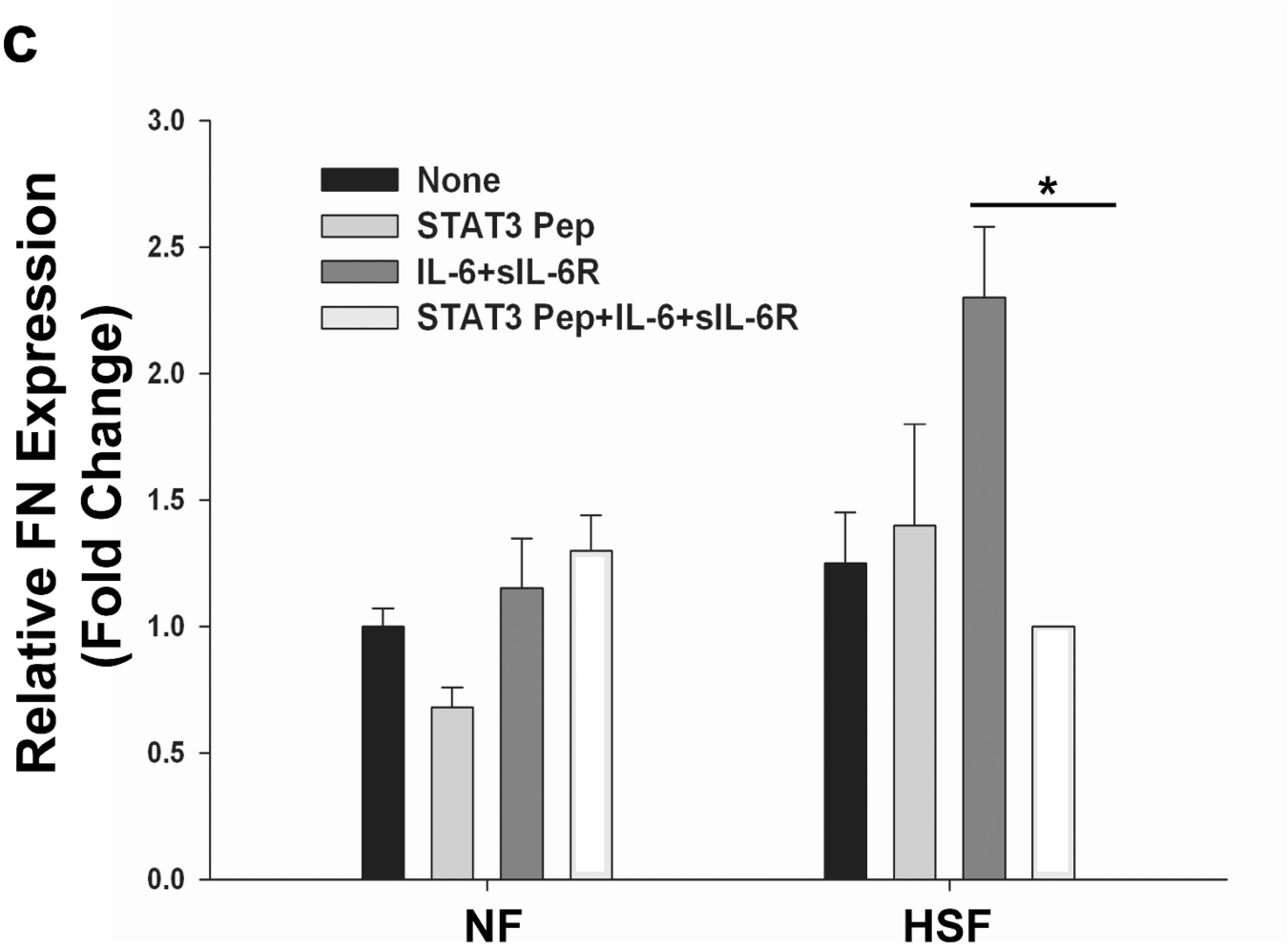




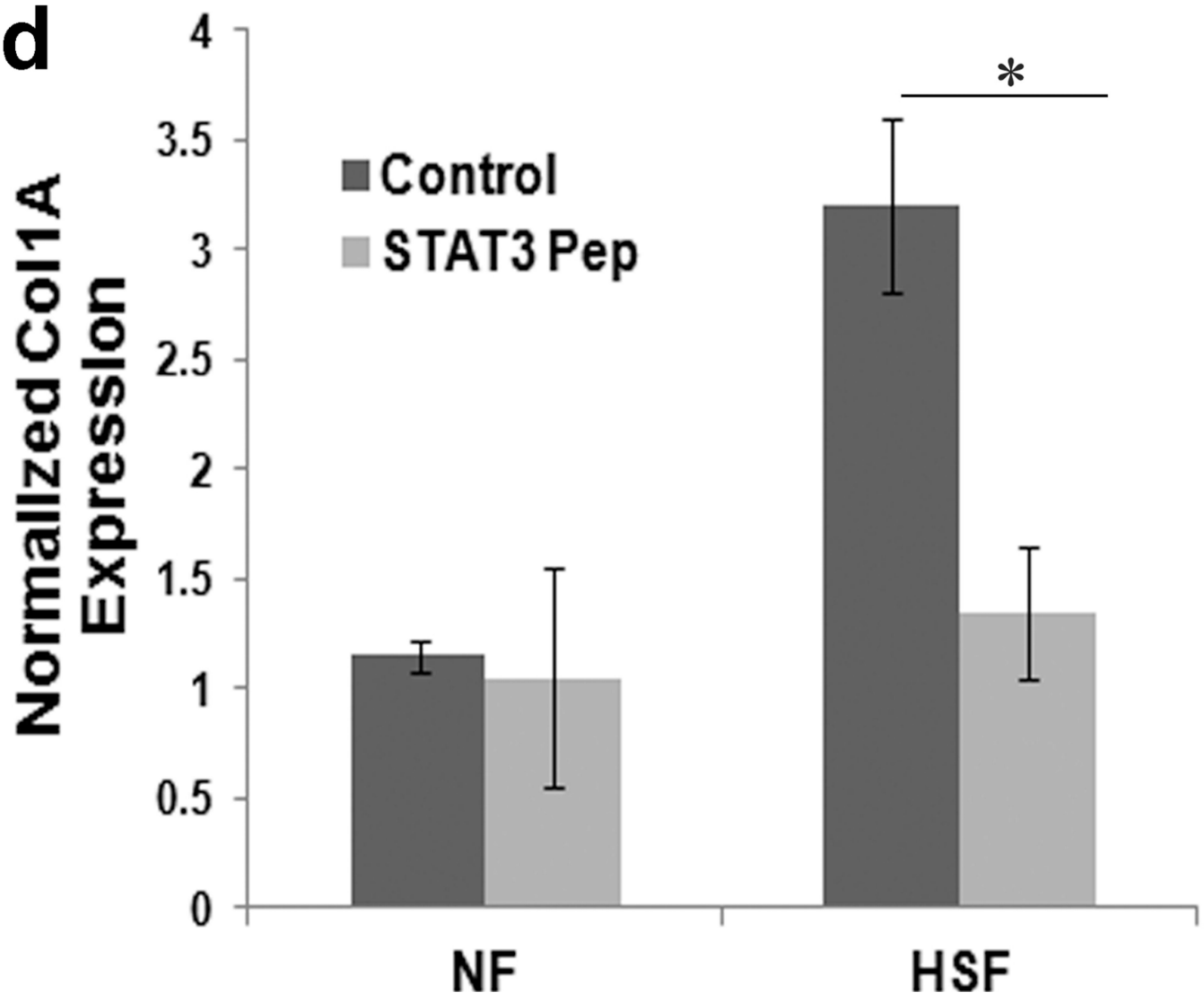


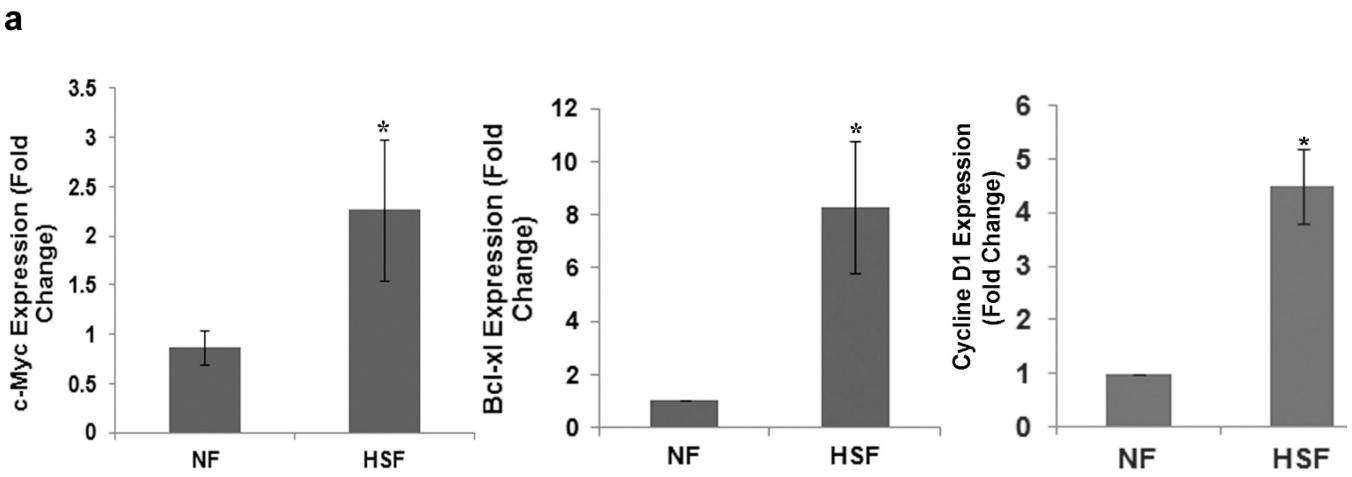
Figure 5.

(a) ECM expression in HSF. NFs and HSFs were stimulated with IL-6•sIL-6Rα and total cellular RNA was subjected to Q-RT-PCR for Col1A2 and FN expression. Shown is fold change mRNA expression relative to GAPDH. *, $p<0.01$.

(b) STAT3 peptide inhibitor attenuates hSOCS3 expression. NF and HSFs were treated with 500 nM STAT3 inhibitor peptide for 6 hoursrs or left untreated. Total cellular RNA (stimulated) was subjected to Q-RT-PCR for hSOCS3 transcript. *, $p<0.01$.

(c) STAT3 peptide inhibitor abrogates FN production in HSF. Cells were treated as in (b) and FN gene expression was measured relative to GAPDH.*, $p<0.05$.

(d) STAT3 peptide inhibitor abrogates Col1A2 production in HSF. Cells were treated as in **(b)** and Col1A2 gene expression was measured relative to GAPDH. *, $p<0.05$.



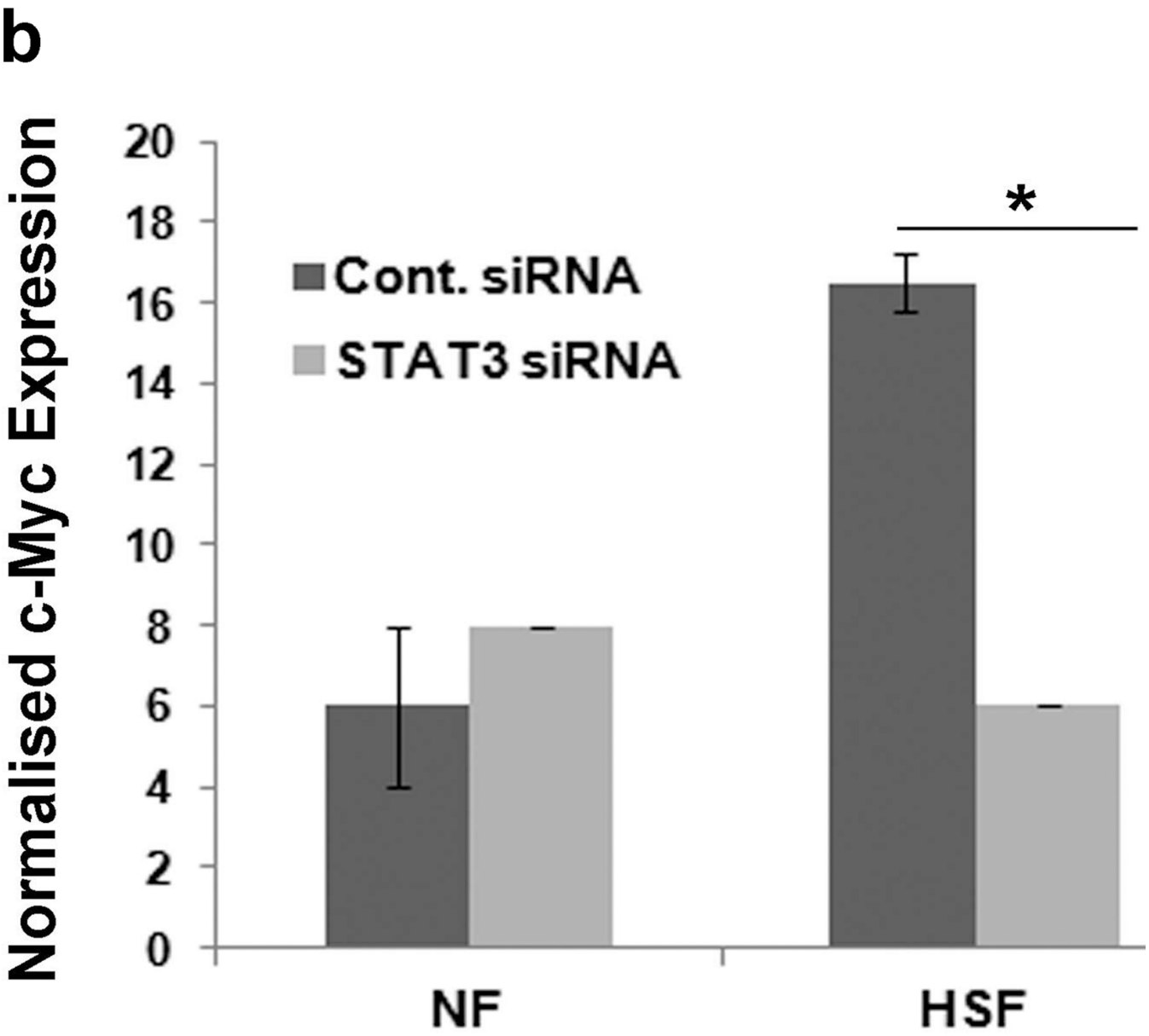


Figure 6.

(a) IL-6 trans-signaling increases expression of cellular proliferation markers in HSF. NF and HSF cells were stimulated with IL-6•sIL-6Rα and total cellular RNA was subjected to QPCR for Cyclin D1, c-Myc and Bcl-XL gene expression. Shown is fold change mRNA expression relative to GAPDH as internal control performed in triplicate. Data represents mean ± SD.*, $p<0.05$, students t test.

(b) STAT3 siRNA inhibits expression of cellular proliferation markers in HSF. NF and HSF cells were transfected with either STAT3 siRNA or control siRNA. 72 h after transfection cells were stimulated with IL-6•sIL-6Rα for 30 min and total cellular RNA was subjected to Q-PCR for c-Myc, Bcl-XL and Cyclin D1 gene expression. *, $p<0.01$, students t test.