

Serum Inter- α -inhibitor Activates the Yes Tyrosine Kinase and YAP/TEAD Transcriptional Complex in Mouse Embryonic Stem Cells*

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Background: Serum contributes to embryonic stem (ES) cell maintenance of pluripotency and activates Yes.

Results: The serum protein Inter- α -inhibitor (I α I) was found to activate the Yes/YAP/TEAD transcription factor pathway and induce expression of Oct3/4 and Nanog in ES cells.

Conclusion: I α I activates key pluripotency pathways.

Significance: I α I may play a significant role in ES cell maintenance and self-renewal.

We have previously demonstrated that the Src family kinase Yes, the Yes-associated protein (YAP) and TEA domain TEAD2 transcription factor pathway are activated by leukemia inhibitory factor (LIF) and contribute to mouse embryonic stem (mES) cell maintenance of pluripotency and self-renewal. In addition, we have shown that fetal bovine serum (FBS) induces Yes auto-phosphorylation and activation. In the present study we confirm that serum also activates TEAD-dependent transcription in a time- and dose-dependent manner and we identify Inter- α -inhibitor (I α I) as a component in serum capable of activating the Yes/YAP/TEAD pathway by inducing Yes auto-phosphorylation, YAP nuclear localization and TEAD-dependent transcription. The cleaved heavy chain 2 (HC2) sub-component of I α I, is demonstrated to be responsible for this effect. Moreover, I α I is also shown to efficiently increase expression of TEAD-downstream target genes including well-known stem cell factors Nanog and Oct 3/4. I α I is not produced by the ES cells *per se* but is added to the cells via the cell culture medium containing serum or serum-derived components such as bovine serum albumin (BSA). In conclusion, we describe a novel function of I α I in activating key pluripotency pathways associated with ES cell maintenance and self-renewal.

Pluripotent stem (PS)² cells, *i.e.* embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, can self-renew indefinitely in culture while retaining the potential to differentiate into any cell type in an organism. To successfully maintain pluripotency and self-renewal, PS cells depend on different signals: Mouse PS (mPS) cells respond to the cytokine leukemia inhibitory factor (LIF) and either serum or bone morphogenic pro-

teins (BMPs) (reviewed in Ref. 1), while human PS (hPS) cells need fibroblast growth factor (FGF) and transforming growth factor (TGF- β , Activin A) (2, 3). The extracellular matrix has also been proven to be important for PS cells, in particular hPS cells, which depend on either feeder cells or extracellular matrix-derived coating for attachment and subsequent survival in culture. The extracellular environment has been linked to both maintenance and directed differentiation (4–8).

LIF has been described to activate several intracellular pathways in mES cells, namely the JAK/STAT3, MAPK, PI3K, and Src-family pathways (reviewed in Ref. 9). Downstream of these pathways an intricate transcriptional network decides the stem cell fate. Some of these transcriptional factors have been designated as stem-cell markers, *e.g.* Nanog, Oct3/4, Tfe3, Sox2, and Esrrb (10). In addition, the YAP-TEAD transcription factor complex has been reported to be important for mES cell self-renewal and maintenance of pluripotency (11, 12). We have shown that LIF signaling through the LIF-receptor activates Yes, which in turn induces nuclear translocation of YAP. Nuclear YAP forms a complex with members of the TEAD transcription factor family (11, 13, 14). Yes, YAP, and TEAD2 are highly expressed in self-renewing mES cells and are down-regulated when cells are induced to differentiate. In addition, the Yes kinase has been shown to suppress differentiation and block embryoid body maturation when overexpressed in mES cells (15). Moreover, TEAD2 can directly associate with the Oct3/4 promoter and activation of the Yes pathway induces, whereas suppression inhibits, Oct3/4 and Nanog promoter activities. In addition to LIF, we have previously demonstrated that Yes can be activated by fetal bovine serum (FBS) but the specific factor(s) in serum responsible for this effect was not identified (13).

In the present study, we have successfully isolated and identified Inter- α -inhibitor (I α I) as one of the components in serum capable of activating the Yes-YAP-TEAD pathway in mES cells. The I α I protein family is a group of protein-glycosaminoglycan-protein complexes that are present in plasma at high concentrations ranging from 0.6 to 1.2 mg/ml in humans (16). They consist of alternate combinations of heavy chains (HC1-HC5)

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² The abbreviations used are: PS, pluripotent stem; YAP, Yes-associated protein; LIF, leukemia inhibitory factor; ES, embryonic stem; iPS, induced PS; I α I, inter- α -inhibitor; ECM, extracellular matrix; HC, heavy chain.

and a light chain called bikunin linked together by a chondroitin 4-sulfate chain, or as unassembled proteins. I α I is the most abundant family member in human serum and consists of the HC1, HC2, and Bk domains. It is mainly produced by the liver and is considered to be inactive until it reaches the target tissue where it is cleaved by TNF-stimulated gene 6 protein (TSG-6), which in turn forms a transient covalent bond with the HCs and transfers them to hyaluronan (HA), a major constituent of the extracellular matrix (ECM) (17).

Up until recently all medium for culturing mouse and human PS cells have included serum or derivatives thereof, such as Knock-Out Serum Replacement (KOSR) or bovine serum albumin (BSA), and thereby also contain I α I proteins. We here show that I α I purified from human plasma, as well as cleaved HC2, but not HC1, activate the Yes/YAP/TEAD2 pathway and induce expression of the pluripotent stem cell transcription factors Nanog and TEAD target genes Oct3/4, Cnr61, and CTGF.

EXPERIMENTAL PROCEDURES

Materials and Cell Lines—Cell lines: Feeder-independent E14 and E14/T (constitutively expressing polyoma large T) mES cells, H181 human ES (hES) cells, K02C human iPS (hiPS) cells, mouse embryonic fibroblasts (MEFs) generated by the Uppsala University Transgenic Facility (Uppsala University), human feeder cell line from human foreskin fibroblasts (HFFs) purchased from ATCC (ATCC-CRL-2429), human kidney cell line (HEKBlue) and human liver tumor cell line (HepG2). Primary antibodies: anti-HC2 (H-90) sc-99107 (Santa Cruz Biotechnology), anti-Oct3/4 (POUF5F1, cl. 7F9.2 (Millipore), anti-YAP #4912 (Cell Signaling), anti-YAP sc-101199 (Santa Cruz Biotechnology), anti- β actin ab8229 (Abcam), anti-Yes 610376 (BD Biosciences), anti-Phospho-Src family (Tyr-416) #2101S (Cell Signaling), anti-P-Ser127-YAP #13008 (Cell Signaling), anti-P-Tyr357-YAP ab62751 (Abcam), and anti-P-Tyr100 #9411 (Cell Signaling). Secondary antibodies: Alexa Fluor 555 goat anti-rabbit (Invitrogen), Alexa Fluor 488 goat anti-mouse (Invitrogen), Alexa Fluor goat anti-rabbit 680 (Millipore), and IRDye 800CW goat anti-mouse (LI-COR Biosciences). Other reagents: Hoechst 33342 (Invitrogen), Odyssey blocking buffer (LI-COR Biosciences), SsoFast EvaGreen Supermix (Bio-Rad), Corning Matrigel hES-qualified (Corning), gelatin 2% (Sigma), protease inhibitory mixture (PIC, Sigma), sodium orthovanadate (Na₃VO₄, Sigma), PD0325901 inhibitor and CHIR99021 inhibitor (both from Selleckchem), LIF (Millipore). Luciferase and β -gal assay reagents: D-Luciferin (Biothema), 2-nitrophenyl β -D-galactopyranoside (Sigma), and adenosine triphosphate (Sigma).

Cell Culture—The E14 and E14/T mES cell lines were cultured on 0,1% gelatin in 10% serum in the absence of feeder cells as previously described (18). Unless stated otherwise, mES cells were plated and grown overnight at 37 °C and 5% CO₂ until 70–80% confluence and then serum-starved for 4–24 h in minimal GMEM-based medium (serum-free medium, here-in called “No FBS”) before treatment with different factors. The mES cells were also cultured in 2i medium (19), a serum-free N2B27 medium supplemented with the MEK inhibitor PD0325901 (1 μ M) and GSK3 inhibitor CHIR99021 (3 μ M), and 1,000 units/ml LIF as described in Ref. 20. The hES cell line

H181 and the hiPS cell line K02C were cultured on Matrigel coating (Corning) in mTESR-1 medium (StemCell Technologies) (21). Mouse embryonic fibroblasts (MEFs), human foreskin fibroblasts (HFFs), human kidney cells HEKBlue, and human liver carcinoma cell line HepG2 were expanded in ATCC-formulated Iscove’s Modified Dulbecco’s medium supplemented with 10% FBS.

Serum Fractionation and Identification of TEAD-activating Fractions—FBS was treated with a mild acetonitrile (ACN) precipitation to separate smaller proteins from its carriers as previously described (22). The supernatant was diluted into 2 M ammonium sulfate containing binding buffer for a modified Blue-Sepharose chromatography, described previously (23) and eluted using 2 M NaCl (Eluate 1) and 1 M arginine (Eluate 2). The eluted fractions were concentrated and dialyzed against phosphate-buffered saline (PBS) using Vivaspin 6 columns (GE Healthcare) and then tested for induction of TEAD activity using luciferase assay (described below). Eluate 1 was further purified using Heparin-Sepharose 6 Fast Flow (GE Healthcare) and eluted stepwise with a NaCl gradient. The eluted fractions were again dialyzed against PBS and tested for their ability to induce TEAD-dependent transcription.

Purification of Human I α I—The isolation of I α I and the heavy chains HC1 and HC2 was performed as described previously (24). Briefly, a side fraction from the commercial production of factor IX (Pharmacia-Upjohn, Stockholm, Sweden) was subjected to gel filtration on a HiPrep 26/60 Sephacryl S-400 HR column using the ÄKTA system (both from GE Healthcare), generating more than 95% pure I α I. For the release of the heavy chains, 2 M NaOH treatment followed by anion exchange chromatography (MonoQ 5/50 GL; GE Healthcare) was performed as described before (25). The fractions were run on an 8% acrylamide SDS-PAGE gel and stained with Coomassie Brilliant Blue followed by mass spectrometry analysis (MS-MALDI-TOF) for assessment of purity. Unless specified differently, protein concentrations were determined by UV measurements and the absorbance coefficients for the protein moieties of I α I, HC1, and HC2 were obtained from Bloom *et al.* (24). The protein solutions were concentrated and dialyzed against PBS on Vivaspin 20 columns and stored at –20 °C.

SDS-PAGE and MS-MALDI-TOF Analysis—Protein samples from the purification were loaded on 8% acrylamide gels. The gels were subsequently stained with Coomassie Blue staining and the selected bands were excised and subjected to MS-MALDI TOF analysis (Mass Spectrometry platform, Uppsala University).

Luciferase and β -Galactosidase Assays—Luciferase expression constructs (final concentration 1–2 μ g DNA/cm²) were introduced into mES cells by transfection with Lipofectamine 2000 (LP2000, Invitrogen) according to the manufacturer’s recommendations. Briefly, cells were incubated in Opti-MEM (Invitrogen), plasmids and LP2000 mixture at 37 °C for 4 h. The transfection was stopped with 10:1 V/V of serum-free medium. The constructs used were pCS GT-IIC-luciferase (GTIIC) (26), pPyCAGIP Nanog, a pGL3-basic vector carrying the 1 kb upstream region of mouse *Nanog* (27), and the pCMV β -gal reference plasmid containing a bacterial β -galactosidase gene. The cells were serum-starved for up to 18 h after transfection

Id1 Activates the Yes/YAP/TEAD Pathway in Mouse Embryonic Stem Cells

and exposed for 6 up to 18 h to the different factors. The different samples were assayed for luciferase and β -galactosidase activities in a microplate luminometer and photometer reader (Wallac VICTOR 1420 Multilabel Counter; Perkin Elmer).

Immunocytochemistry (ICC)—Cells were fixed with cold 4% paraformaldehyde (Sigma) for 15 min and then blocked with PBS 0.5% BSA and 0.3% Triton-X100 (Sigma). The cells were incubated at 4 °C overnight with primary antibodies anti-YAP (1:300) and anti-Oct3/4 (1:300), and subsequently incubated with Alexa Fluor secondary antibodies and co-stained with Hoechst (DAPI). Coverslips were mounted using fluoromount (Sigma), and analyzed with inverted confocal fluorescent microscopy (LSM700, Zeis). YAP nuclear localization was measured using ImageJ freeware (NIH). Shortly, DAPI staining was used to determine the limits of nuclear localization and Oct3/4 staining confirmed pluripotent stem cell colonies. Relative YAP nuclear staining was calculated by dividing YAP nuclear staining relative to area divided by cytoplasmic staining relative to area.

Immunoprecipitation and Western Blotting—Cells were incubated 15 min with 100 μ M Na_3VO_4 and harvested in lysis buffer as previously described (13). The lysates were sonicated, and total protein concentration was measured using BCA Protein Assay kit (Pierce). 10 μ g anti-Yes antibody (BD Biosciences) or anti-YAP antibody (Santa Cruz Biotechnology) were coupled to Protein A Dynabeads according to the manufacturer's recommendations using the Immunoprecipitation Dynabeads Protein A kit (Life Technologies) and then incubated end-over-end with 1 mg of protein extract overnight at 4 °C. The bound proteins were eluted in blotting loading buffer containing SDS and β -mercaptoethanol. Immunoprecipitated fractions and total lysates were run on 7.5% acrylamide gels under denaturing conditions, and the proteins were transferred to an Immobilon-FL membrane (Millipore). The membranes were blotted with anti-phospho-Src family (Tyr-416, 1:500), anti-phospho tyrosine (P-Tyr100, 1:500), total YAP (1:250), β actin (1:5000), total Yes (1:1000), P-Ser127 YAP (1:1000), or P-Tyr357 YAP (1:500). The membranes were then incubated with Alexa Fluor anti-rabbit 680 (1:1000) and IRDye 800CW anti-mouse (1:1000). Immunosignals were imaged using an Odyssey fluorescent imaging scanner and band intensity quantification was analyzed using Odyssey 2.1 software (LI-COR Biosciences).

qPCR and RT-PCR—Total RNA was extracted and purified with Qiagen RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The cDNA was reverse-transcribed from 1 μ g of total RNA using iScript (Bio-Rad). First-strand cDNAs were amplified by Quantitative real-time PCR using the MiniOpticon Real-Time PCR Detection System (Bio-Rad), SsoFast Evagreen Supermix and specific primers. The average C_t value for each gene was normalized against GAPDH or 18 S ribosomal RNA (18 S) gene expression and the comparative C_t value (fold change) was calculated using $2^{-\Delta\Delta C(t)}$. Transcript level comparison was based on primer efficiency estimated from five-point dilution curves and used for comparative C_t computation according to the PFAFFL method (28). For RT-PCR the first-strand cDNAs were amplified using the SsoFast Evagreen Supermix (Bio-Rad) and specific primers. The PCR

products were electrophoresed on a 2% (w/v) agarose gel with GelRed (Biotum) staining, and photographed using the UVP BioImaging system (Bio-Rad). The PCR primers used are: mouse HC1 (forward: aggtctgacgttcgggctcgt, reverse: ggcccagagcctctccacatga), mouse HC2 (forward: agtgatcttgggaactggccca, reverse: acagtgtccgtctcctctccgg), mouse TSG-6 (forward: cgtccacggcttttaggaagatac, reverse: agcctccagccgtgacggat), mouse Oct3/4 (forward: gatgctgtgagcccaaggcaag, reverse: ggctctgatcaacagcatcac), mouse Nanog (forward: cttccacctattaagg-tgcttc, reverse: tggcat cggttcatcatggtac), mouse Cyr61 (forward: cagctc actgaagaggcttct, reverse: gcgtgcagagggtgaaaa), mouse CTGF (forward: cttctgcgatttcggctcc, reverse: tacaccgaccaccgaaga), mouse 18 S (forward: agtcctgccctttgtacaca, reverse: gatccga- gggcctcactaac), human HC1 (forward: gcctgg-tgttatcctgagcc, reverse: cggggttcagcgtaatgctc), human HC2 (forward: ctcacctaagcccacggac, reverse: ggcttctcagggtccttcc), human TSG-6 (forward: aagcaggtctggcaatatacagc, reverse: atccatccagcagcacagacatga), human GAPDH (forward: gaaggtg-aaggtcggagtc, reverse: gaagatggtgatgggatttc).

Statistical Analysis—Experiments were performed at least in three independent experiments, and data are presented as mean \pm S.E. When applicable, statistical analysis was done using One-way ANOVA with Dunnett's test on GraphPad Prism version 5.00d for Mac (GraphPad Software, San Diego, CA), * = $p < 0.05$, ** = $p < 0.001$, and *** = $p < 0.001$.

RESULTS

FBS Activates TEAD-dependent Transcription in a Dose and Time-dependent Manner—We have previously shown that FBS induces auto-phosphorylation activity of the Src-family member Yes (13). To determine whether FBS also activates TEAD-dependent transcription, mES cells were transfected with a reporter gene construct expressing the firefly luciferase gene driven by the human chorionic somatomammotropin (CS) promoter with multiple (24) copies of the TEAD-binding GTIIC enhancer (pGTIIC-Luc), as previously described (11). The TEAD-binding enhancer region binds all four TEAD family transcription factors (TEAD1-TEAD4) and therefore measures induction of TEAD family-dependent transcription (26). In a previous report, we demonstrated only minimal luciferase activity in mES cells transfected with the control luciferase construct containing only the CS promoter (11). After transfection, the mES cells were serum and LIF-starved, stimulated with increasing concentrations of FBS and harvested at different time points. The TEAD-dependent luciferase expression was subsequently analyzed. Total protein values were used to normalize luciferase levels. As shown in Fig. 1, A and B, FBS significantly activates TEAD-dependent transcription in a dose and time-dependent manner. The highest TEAD activity was achieved with 5% FBS and for 6-h exposure time, and therefore all subsequent experiments to assess activation of TEAD-dependent transcription were performed using these conditions, unless stated otherwise.

BMP4 Has No Effect on TEAD-dependent Transcription—BMP4 can replace FBS in mES cell cultures (29). It has also been shown that YAP binds to SMAD1 in response to BMP signaling and contributes to mES cell maintenance of self-renewal and pluripotency by inducing *Id* gene transcription (30). Therefore,

I α 1 Activates the Yes/YAP/TEAD Pathway in Mouse Embryonic Stem Cells

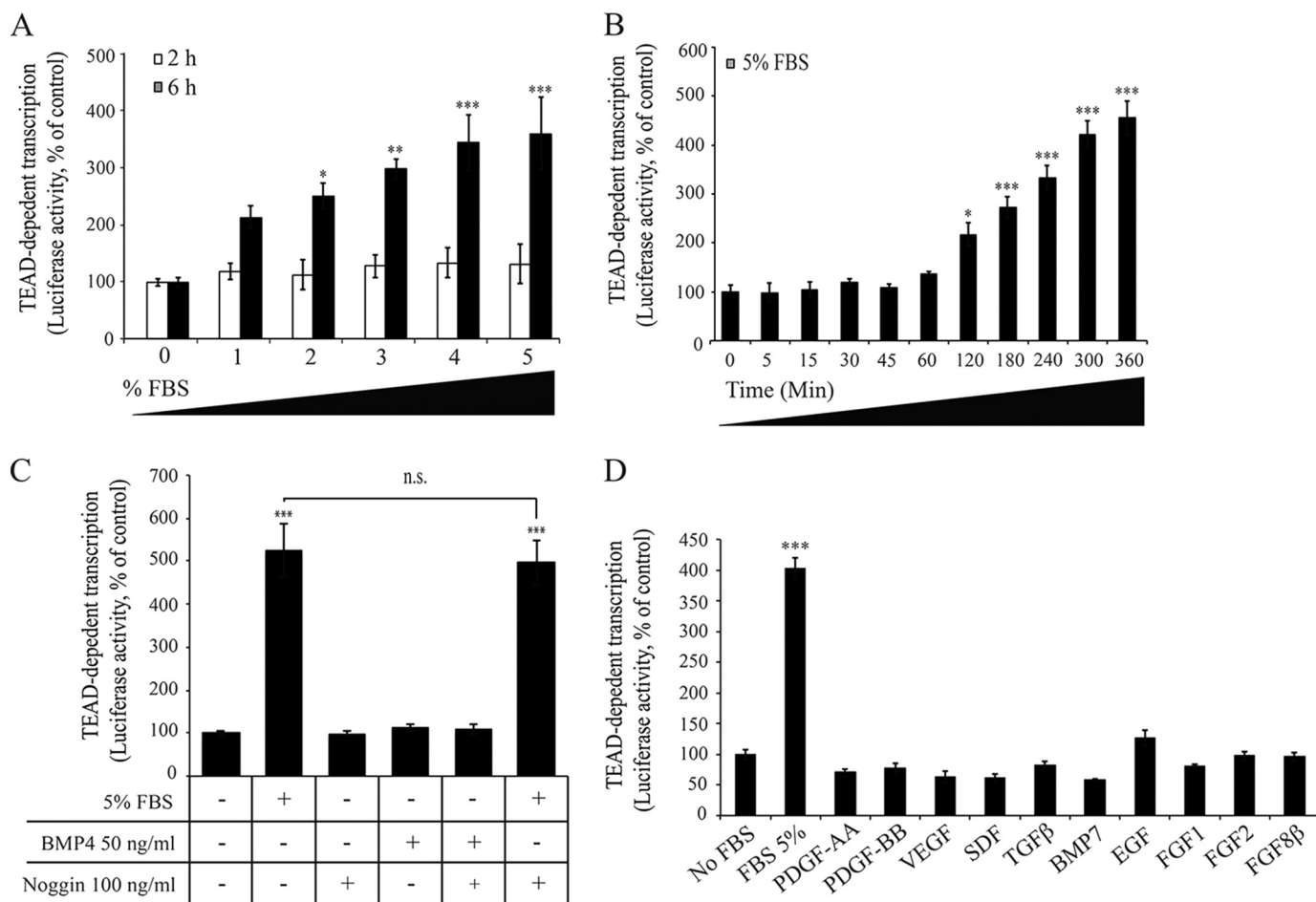


FIGURE 1. FBS activates TEAD-dependent transcription in mES cells in a dose- and time-dependent manner while different commercial growth factors have no effect on TEAD activity. TEAD-dependent transcription was measured as luciferase activity of mES cells transfected with pGTIIIC-Luc construct and was normalized to total protein content. Cells were LIF- and serum-starved for 24 h and exposed to: *A*, increasing concentration of FBS for 2 h (white bars) or 6 h (black bars); *B*, 5% FBS for different time periods; *C*, 5% FBS, 50 ng/ml Noggin, and/or 100 ng/ml BMP-4 for 6 h; or *D*, 5% FBS, 10 ng/ml PDGF-AA, 10 ng/ml PDGF-BB, 50 ng/ml VEGF, 10 ng/ml SDF, 5 ng/ml TGF- β , 100 ng/ml BMP-7, 10 ng/ml FGF1, 10 ng/ml FGF2, or 10 ng/ml FGF8 β for 6 h. All bars represent mean \pm S.E.

we considered BMP4 as a potential component of serum capable of activating TEAD transcription. However, BMP4 did not induce TEAD-dependent transcription. Moreover, the addition of the BMP inhibitor Noggin did not inhibit the capacity of FBS to induce TEAD, suggesting that another factor present in serum activates TEAD-dependent transcription (Fig. 1C).

Different Commercial Growth Factors Do Not Affect TEAD-dependent Transcription—To assess whether the TEAD activity could be induced by other growth factors present in serum, we tested a panel of commercially available growth factors for their ability to affect TEAD family-dependent transcription, in serum-starved cells. However, no noticeable effect on TEAD-dependent transcription was detected after stimulation with the different factors, except for EGF, which caused a small yet insignificant increase in TEAD dependent-transcription (Fig. 1D).

I α 1 Is a Component in Serum Able to Increase TEAD Activity in mES Cells—Because none of the tested growth factors affected TEAD-dependent transcription, a reverse approach was initiated with the aim of isolating and identifying one or more components in serum that can induce TEAD-dependent transcription. Thus, FBS was separated by chromatogra-

phy as described under “Experimental Procedures,” and the eluted fractions were tested for their ability to induce TEAD-dependent transcription. Mouse ES cells co-transfected with pGTIIIC-Luc and pCMV β -gal constructs were then serum- and LIF-starved and subsequently stimulated with the eluted fractions. The luciferase activity was normalized to the β -gal transcription level to remove transfection rate variations between samples and overall activation induced by the complex mix of growth factors, cytokines, and other molecules present in FBS. The fractions were also separated with SDS-PAGE and stained with Coomassie Blue to assess protein content. Fig. 2A shows the stained gels and the respective fraction’s ability to induce TEAD-dependent transcription. Eluate 1 from the first Blue-Sepharose chromatography step was found to induce the highest level of TEAD activation, and was therefore used for further separation by heparin chromatography. The eluted fractions were again analyzed for TEAD-dependent activation as described above (Fig. 2B).

By correlating band intensity with luciferase activity we identified two protein band bands that were stronger in the fractions with higher luciferase activity and hypothesized that any of these bands may contain a protein that is able to activate TEAD (Box

I α I Activates the Yes/YAP/TEAD Pathway in Mouse Embryonic Stem Cells

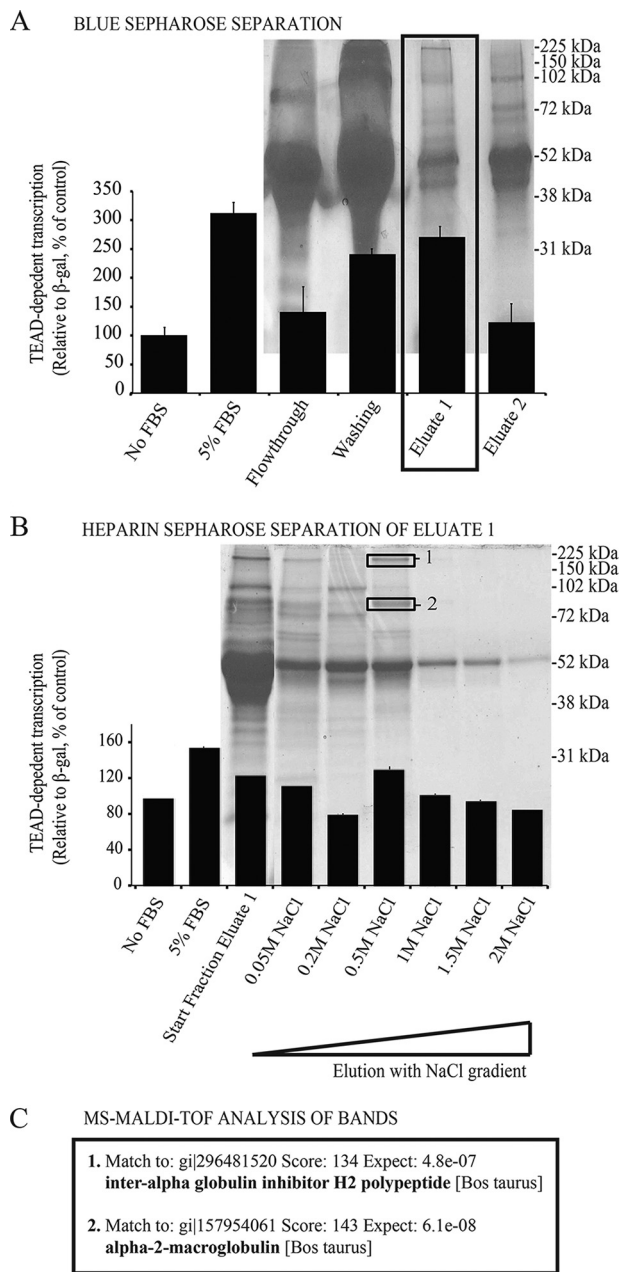


FIGURE 2. Fractionation of serum and identification of serum proteins capable of TEAD activation in mES cells. A, FBS was treated with 20% acetonitrile and subjected to a Blue-Sepharose chromatography. The different fractions were analyzed by SDS-PAGE and Coomassie Blue staining and added to LIF and serum-starved mES cell cultures for 6 h. Induction of TEAD-dependent luciferase activity was then measured as compared with untreated control. 5% FBS was used as positive control. B, Eluate 1, induced the highest luciferase activity and was therefore selected for further fractionation by heparin chromatography. The different eluted fractions were again subjected to SDS-PAGE and Coomassie Blue staining and tested for their ability to induce TEAD-dependent transcription. Bars represent mean \pm S.E. The luciferase activity was compared with the protein patterns of the different fractions and two bands (boxes 1 and 2) were identified as possible TEAD-activating proteins. C, bands in box 1 and 2 were then excised, and the protein identity was determined by MS-MALDI-TOF analysis.

1 and 2 in Fig. 2B). The bands were therefore excised and subjected to MS-MALDI-TOF analysis. The protein band in Box 1 was identified as Inter- α -inhibitor heavy chain 2 polypeptide B (HC2, *Bos taurus*) and the protein band in box 2 as α -2-macroglobulin (*Bos taurus*) (Fig. 2C). Because of its key function in

oocyte formation, its presence in amniotic fluid and its recently reported capacity to induce cell signaling (31–33), I α I-HC2 was considered to be the most likely candidate for activating TEAD, and was therefore selected to be studied in more detail.

I α I Induces TEAD-dependent Transcription in a Dose-dependent Manner—To assess whether I α I can activate TEAD-dependent transcription, human I α I was purified from a side-fraction of human plasma, as described in Ref. 24. Moreover, the two heavy chains, HC1 and HC2, were released by alkaline treatment and purified with a MonoQ chromatography (24). Separation of the purified proteins on an SDS-PAGE gel and MS-MALDI-TOF analysis confirmed more than 95% purity (Fig. 3A).

Purified human I α I was then tested for its ability to induce TEAD-dependent transcription as described above. As shown in Fig. 3B, purified I α I did indeed significantly activate TEAD in a dose-dependent manner. Moreover, the β -galactosidase activity in I α I exposed cells was maintained at a similar level as the serum-starved cells, while the luciferase TEAD-dependent activity increased, suggesting a specific effect on TEAD rather than a general effect on transcription or cell proliferation. In contrast, FBS increased both β -galactosidase and TEAD-dependent transcription (results not shown).

I α I and the Cleaved I α I-HC2 Globular Domain Induce TEAD-dependent Transcription and Nanog Promoter Activation—I α I is known to be cleaved *in vivo* by TSG-6 and the cleaved heavy chains are then covalently bound to hyaluronan, where they modify the properties of the extracellular matrix. Moreover, the released heavy chains have been shown to induce effects of their own, such as inhibiting the migration properties of a malignant glioma cell line *in vitro* (33, 34). Therefore, purified HC1 and HC2 domains were independently assessed for their ability to activate TEAD family-dependent transcription. HC2 activated TEAD-dependent transcription in a similar manner as the whole I α I protein, while HC1 did not induce any effect on TEAD-dependent transcription (Fig. 3C).

We have previously shown that the Yes/YAP/TEAD pathway can activate the Nanog-promoter via induction of Oct 3/4 (11). Thus, Nanog-promoter activity was assessed by co-transfecting mES cells with a Nanog-promoter luciferase reporter construct and the pCMV β -gal construct. Cells were then serum-starved and exposed to FBS, I α I or HC2 for 18 h. FBS, I α I, and HC2 all induced significant Nanog-promoter activity (Fig. 3D). Moreover, 24 h exposure to FBS, LIF, or I α I in serum-free medium increased Nanog mRNA levels as assessed by qPCR (Fig. 3E).

I α I Induces Transcription of TEAD Target Genes *Cyr61*, *CTGF*, and *Oct3/4*—To further investigate the effect of I α I on TEAD-dependent transcription, mES cells were exposed to FBS, LIF, and I α I for 24 h and subsequently subjected to qPCR analysis for *CTGF*, *Cyr61*, and *Oct3/4*, which are all reported target genes for the YAP/TEAD complex. *CTGF*'s promoter region contains several TEAD-binding elements (35–37), *Cyr61* has been shown to be up-regulated by YAP/TAZ in several microarray studies (35–37) and *Oct3/4* has been reported to be a direct target of the YAP/TEAD complex (11, 12). I α I consistently and significantly increased the expression of all three target genes, similarly to FBS (Fig. 3, F, G, and H).

IαI Activates the Yes/YAP/TEAD Pathway in Mouse Embryonic Stem Cells

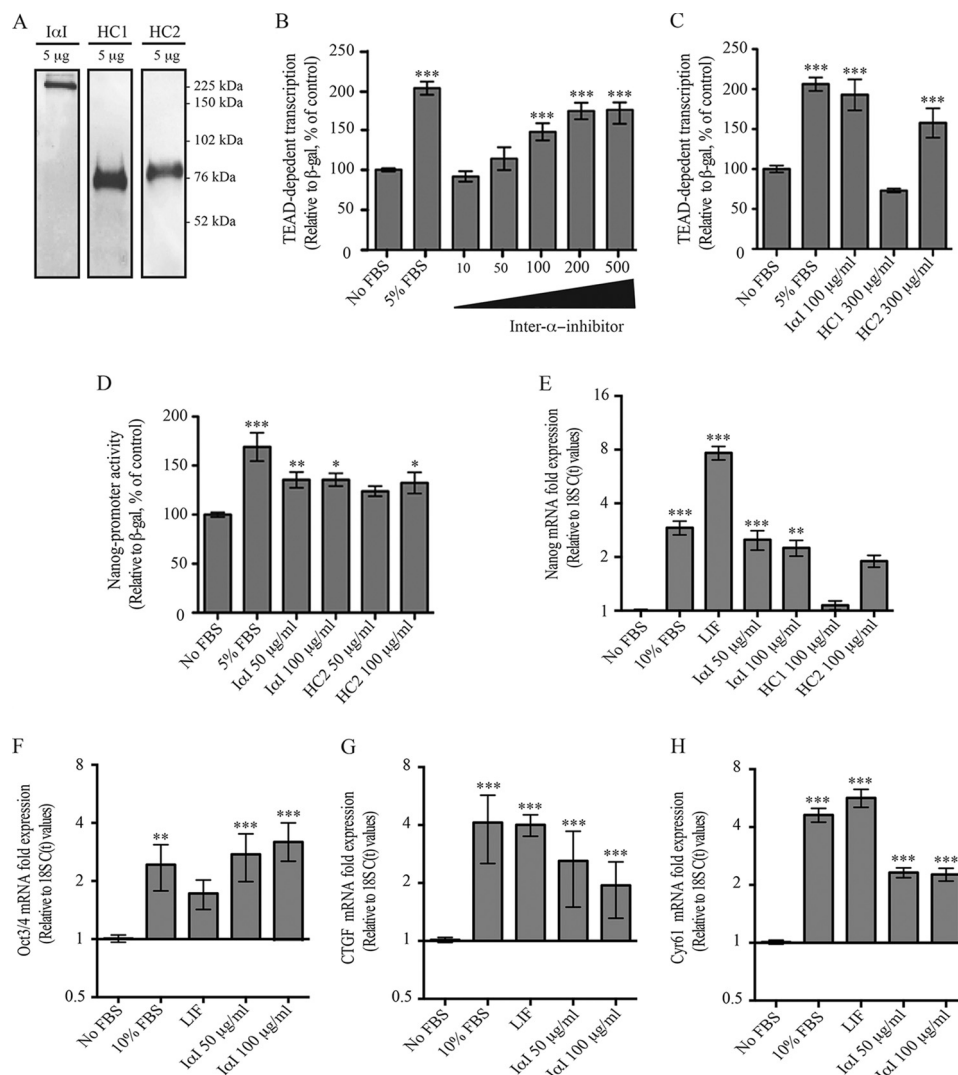


FIGURE 3. IαI and HC2 induce TEAD-dependent transcription, Nanog promoter activity, and mRNA expression of Nanog and TEAD target genes. *A*, SDS-PAGE and Coomassie Blue staining of human IαI, HC1 and HC2 purified from human plasma. *B*, TEAD-dependent transcription in serum-starved E14 mES cells treated with FBS and increasing concentrations of IαI for 6 h. *C*, TEAD-dependent transcription in mES cells in response to FBS, IαI, HC1, and HC2 for 18 h. *D*, Nanog-promoter activity in E14 mES cell line in response to IαI and HC2 for 18 h. *E*, qPCR analysis of Nanog (*F*) Oct3/4 (*G*), CTGF and (*H*) Cyr61 levels in serum-starved E14 mES cells treated with LIF, FBS, and IαI for 24 h. All bars show mean ± S.E. mRNA levels relative to 18S C(t) values and normalized to the serum-starved negative control.

IαI Activates Yes and YAP—Upon activation, YAP translocates to the nucleus where it can form a complex with members of the TEAD family and induce or enhance transcription of various genes (14). Thus, to further investigate the effect of IαI on the YAP-TEAD pathway, the YAP nuclear localization in response to different factors was assessed. Increased relative nuclear staining was measured in mES cells treated with LIF, IαI, HC2, and hyaluronan, as compared with serum-starved mES cell colonies; while bFGF and HC1 did not exhibit any significant effect (Fig. 4, *A* and *B*). Interestingly, the 2i inhibitor mixture containing CHIR99021 and PD0325901, which is known to stimulate stem cell factors and has been described to accomplish lower grades of spontaneous differentiation in culture, also increased YAP nuclear localization, further linking YAP nuclear localization to stem cell maintenance and self-renewal (19, 38).

To further investigate whether IαI can activate the Src family kinase Yes in ES cells, mES cells were serum starved followed by

stimulation with 100 μg/ml IαI for 40 min. Cell lysates were subjected to immunoprecipitation with anti-Yes antibody and subsequent Western blot analysis for total and tyrosine 416-phosphorylated Yes. The latter recognizes the auto-phosphorylation site in the activation loop of the kinase domain of all the Src family of non-receptor tyrosine kinases, which is necessary for full catalytic activity. Our results show that IαI indeed induces an early increase in Yes auto-phosphorylation, suggesting that IαI at least partly signals via activation of Yes (Fig. 4C).

Phosphorylation of YAP on serine 127 induces binding to 14-3-3 and subsequent cytoplasmic retention (reviewed in Ref. 39). In contrast, tyrosine 357 phosphorylation leads to YAP activation and translocation to the nucleus where it forms a transcriptionally active complex with members of the TEAD family (14, 40). In order to examine the effect of IαI-stimulation on YAP phosphorylation, mES cells were serum starved for 5 h and subsequently exposed to FBS or IαI for 2 h. YAP phosphorylation was assessed by Western blotting of YAP-immunopre-

IαI Activates the Yes/YAP/TEAD Pathway in Mouse Embryonic Stem Cells

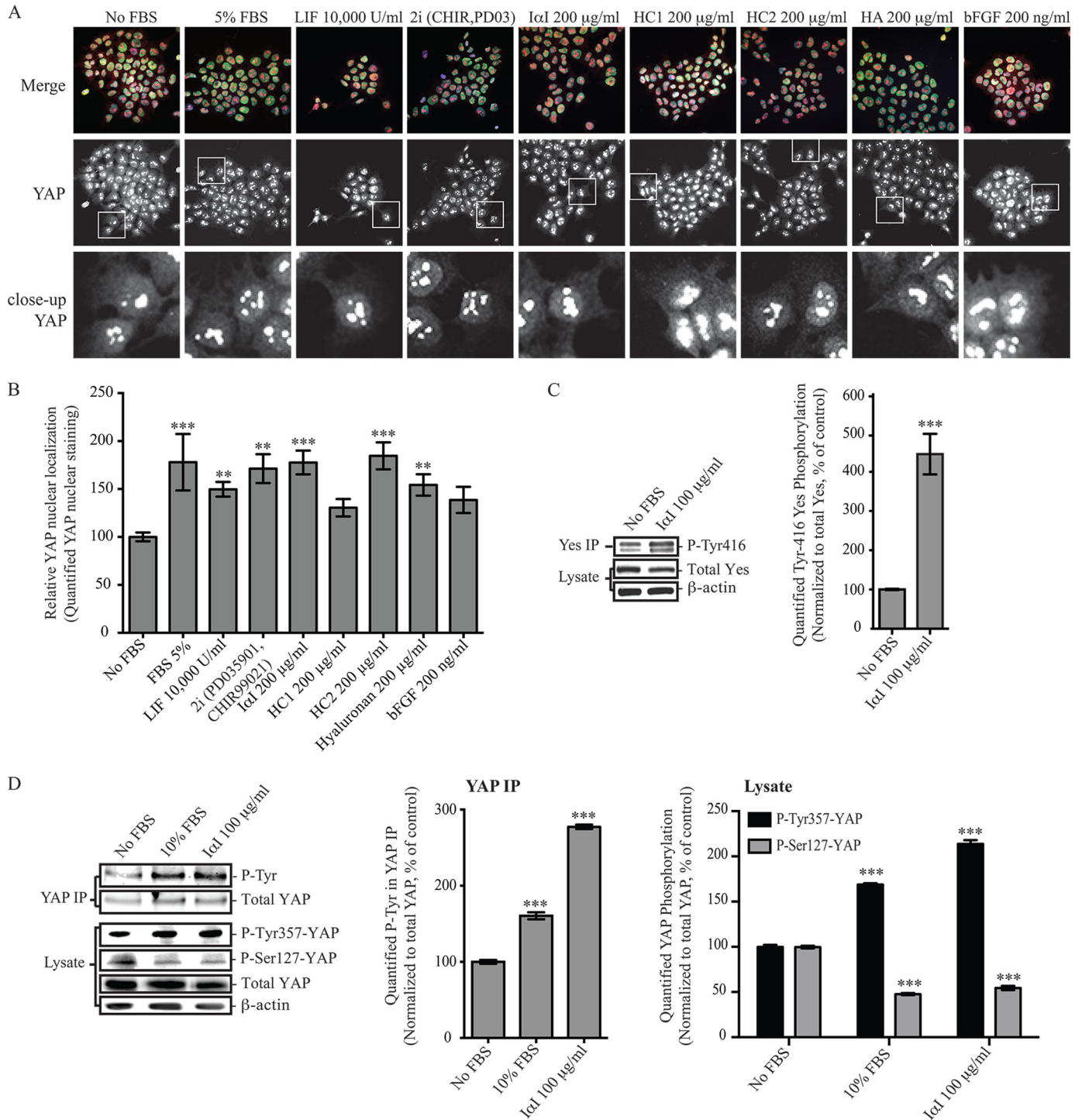


FIGURE 4. IαI induces YAP nuclear localization and Yes tyrosine phosphorylation. *A*, representative images of cell densities for the YAP nuclear localization assay, E14 mES cell line colonies were immunostained for Oct3/4 (green), YAP (red), and DAPI (blue) and analyzed using confocal microscopy. *B*, relative YAP nuclear localization as assessed using immunocytochemistry staining and ImageJ fluorescence quantification in LIF and serum-starved E14 mES cells treated with different factors for up to 40 min. Bars represent YAP nuclear staining relative to the serum-starved control (No FBS). *C*, Yes tyrosine auto-phosphorylation in response to IαI. Lysates were prepared from LIF- and serum-starved E14/T mES cells treated with 100 μg/ml IαI for 40 min and subjected to anti-Yes immunoprecipitation and Western blotting analysis for auto-phosphorylated Yes, total Yes, and β-actin. *D*, YAP tyrosine and serine phosphorylation in response to FBS and IαI for 2 h. YAP immunoprecipitates and total lysate were blotted for total YAP, P-Tyr100, P-tyr357 YAP, P-Ser127 YAP, and β-actin. Western blot bands were quantified using Odyssey software (LI-COR), bars represent mean ± S.E.

precipitated and total cell lysate. As shown in Fig. 4D, IαI induced a significant increase in tyrosine 357 phosphorylated YAP concurrent with decreased levels of serine 127 phosphorylated YAP, providing additional evidence that IαI induces activation and nuclear translocation of YAP.

IαI Is Present in ES Cell Medium and Is Transferred to the ECM by TSG-6—ES cells grown in serum-containing medium are exposed to high IαI concentrations, but there are also new serum-free medium available for successful ES culture that are based on serum-derived components such as KOSR and BSA.

Iα1 Activates the Yes/YAP/TEAD Pathway in Mouse Embryonic Stem Cells

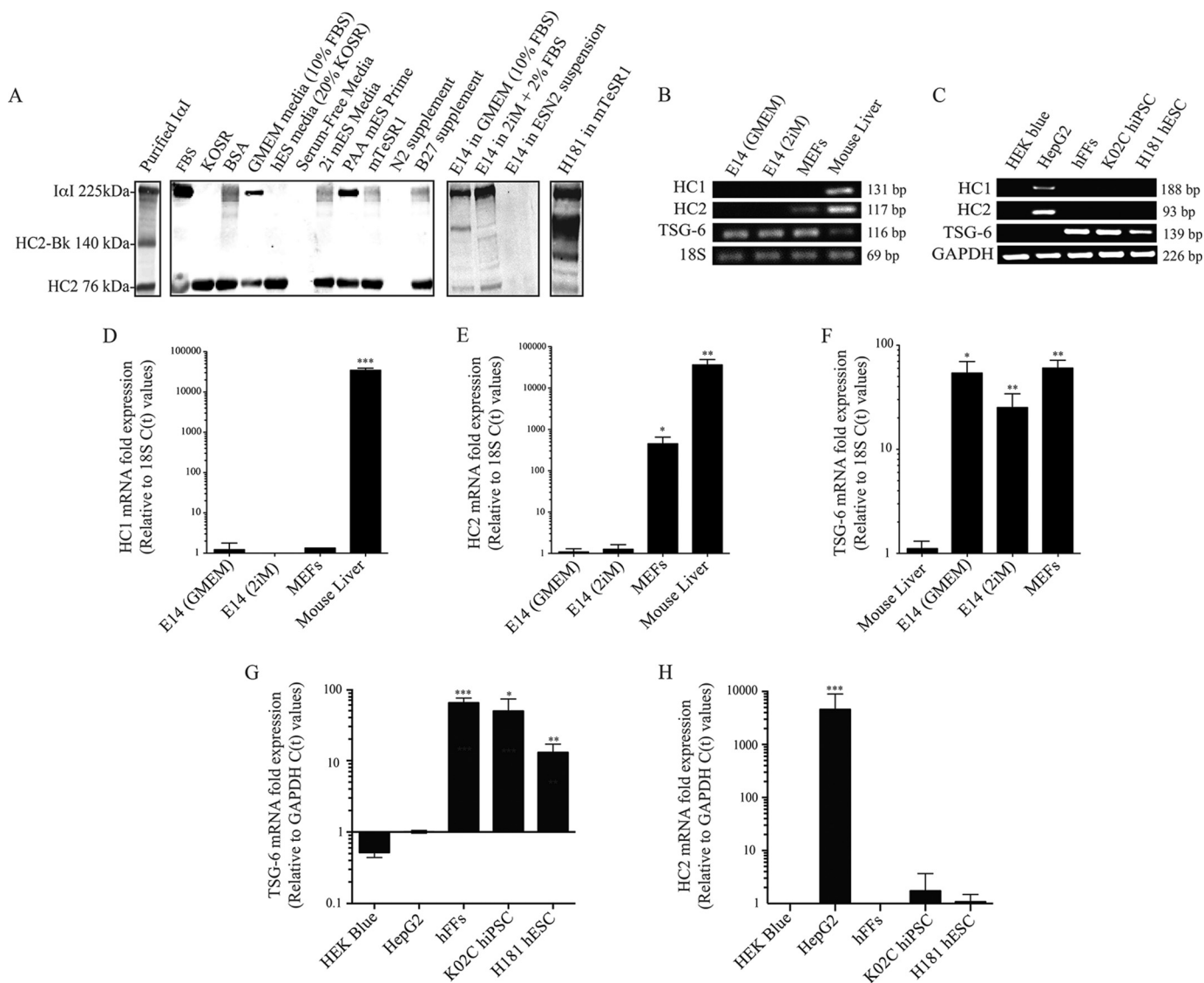


FIGURE 5. Iα1 is present in ES cell medium. Human and mouse PS cells and feeders do not produce significant levels of HC1 and HC2 while they do produce TSG-6 mRNA. A, Western blot analysis for HC2 of purified Iα1, FBS, FBS-derivatives and medium formulations as well as of total lysates from E14 mES cells cultured in different medium and the H181 hES cells cultured in mTeSR on Matrigel coating. B, RT-PCR analysis of HC1, HC2, TSG-6, and 18 S mRNA in mES cells cultured in complete medium and 2i medium, MEFs and Mouse liver tissue sample. C, RT-PCR analysis of HC1, HC2, TSG-6, and GAPDH mRNA in HEKBlue human kidney cells, HepG2 human liver tumor cells, H181 hES cells, K02C human iPS cells, and CRL-2429 human feeder cells. qPCR analysis of (D) HC1, (E) HC2, and (F) TSG-6 mRNA normalized to 18 S C(t) values in mES cells cultured in different medium, MEFs and mouse liver. qPCR analysis of (G) TSG-6 and (H) HC2 mRNA normalized to GAPDH C(t) values in HEKBlue, HepG2, CRL-2429, H181, and K02C cells.

To assess the content of Iα1 in different ES medium, various commercial medium supplements and formulations were analyzed by Western blotting for the presence of Iα1 and/or HC proteins. All serum-derived components, including KOSR and BSA, contain Iα1 or Iα1 sub-components such as HC2, as do all medium containing any of those serum-derived products (Fig. 5A). Medium without serum-derived additives (N2, serum-free medium) does not contain any traces of Iα1. Total lysates from mouse and human ES cells expanded in medium with high levels of Iα1 (FBS containing GMEM and 2i medium, and BSA containing mTeSR medium) contained either whole Iα1 or HC2. However, cells cultured in medium without Iα1 (ESN2 suspension culture, based on N2 supplement in DMEM/F12 and no serum-derived components) exhibited no detectable Iα1 (Fig. 5A).

To assess whether Iα1 is produced by ES cells *per se* or if it is only introduced to the cells via the medium, HC1, HC2 and

TSG-6 mRNA levels were analyzed using RT-PCR (Fig. 5B). HC1 mRNA was undetectable in MEF feeder cells and mES cells expanded in two different culture medium. HC2 mRNA was also undetectable in mES cells while a low level of HC2 transcript was observed in MEF feeder cells. As expected, mouse liver tissue, the positive control, expresses high levels of both HC1 and HC2 transcripts. In contrast, TSG-6 mRNA was found in both mES cell cultures and the MEF feeder cell line, while no detectable TSG-6 mRNA levels were found in mouse liver (Fig. 5B). The different mES cells, MEFs and liver samples were also subjected to qPCR, and the results confirmed the RT-PCR data (Fig. 5, D, E, and F).

The human ES cell line H181, the human iPS cell line K02C, the human feeder cell line hFF, the human liver cell line HepG2, and the human kidney cell line HEKBlue were also analyzed by RT-PCR and qPCR for their ability to produce HC2, HC1, and

IαI Activates the Yes/YAP/TEAD Pathway in Mouse Embryonic Stem Cells

TSG-6 (Fig. 5, C, G, and H). As expected, neither the human kidney negative control cells, the human ES cells nor the human iPS cells express any HC2 mRNA, nor did the hFF feeder cell line. However, as expected the human liver cell line expresses high levels of both HC1 and HC2, while no TSG-6 mRNA was detected. Similar to mES cells, the human PS cell lines and the human feeder cells express high levels of TSG-6 mRNA (Fig. 5, C and G), further supporting the idea that IαI is supplemented, and that the cells capacity to produce TSG-6 confers them with the ability to transfer the HCs to the hyaluronan chains present in their ECM.

DISCUSSION

Serum supports attachment, survival, and proliferation of many cell types, including pluripotent stem cells. Many cytokines and extracellular matrix proteins have been identified in serum as key factors for the induction of proliferation and attachment, but many others remain to be studied for their effect on cell culture. Even though stem cell research is moving toward serum-free conditions, novel medium that are described as serum-free usually still contain traces of serum-derived components through supplements such as BSA, and therefore contain IαI proteins, as suggested by our results. In this study, we establish that IαI, a major component of serum, induces a signaling pathway known to activate the key stem cell factors Oct3/4 and Nanog.

We have previously shown that LIF activates the Src family kinase Yes, the Yes-associated protein (YAP) and the family of TEAD transcription factor, and that TEAD in its turn can enhance transcription of other stem cell factors such as Oct3/4 and Nanog. For the first time we show that an ECM protein can also activate this pathways in a similar manner. IαI has traditionally been described as a structural component of the ECM and the HCs are the only proteins that have been demonstrated to bind covalently to HA (41). IαI has been described to be necessary for the cumulus formation of the ovary (31) and to play an important role in tissue repair (34, 42, 43). Moreover, an isoform of IαI has been reported to be produced by the amniotic membrane, and the hyaluronan-HC (HC·HA) complex is present in amniotic fluid, giving evidence of TSG-6 mediated transfer of HCs to hyaluronan in amniotic tissue (32, 44). These data suggest that the HCs play a role during embryonic development and may also be important for ES cell maintenance. Neither mouse nor human embryonic stem cells express IαI proteins as assessed by HC1 and HC2 mRNA expression. However, they express TSG-6, supporting the hypothesis that IαI is introduced to the cells through the medium and that the HCs are transferred to the stem cell's ECM and hyaluronan through TSG-6-mediated transfer.

IαI also induces signaling events that promote self-renewal of mES cells, such as Yes-activating tyrosine auto-phosphorylation, YAP activation by tyrosine phosphorylation and nuclear localization, TEAD family-dependent transcription, Nanog-promoter activity and mRNA production of the TEAD target genes Oct3/4, Cyr61, and CTGF.

Human serum contains an amount of IαI ranging from 0.6–1.2 mg/ml (16). Thus, if estimating a concentration of 1 mg/ml in bovine serum; 5% FBS would account for 50 μg/ml. Never-

theless, a higher IαI concentration, 100 μg/ml, was required in some experiments to achieve significant TEAD response. The reason may be that serum contains other components that add to the TEAD-activating effect, or it could be a side-effect of compromised IαI stability since we have observed that purified IαI exhibits a tendency to aggregate and partly degrade over time.

The HC1 and HC2 domains are quite conserved between different species, and the mouse, human and bovine HC proteins share 80–85% amino acid identity. This concurs with our data showing that both bovine and human IαI induce similar effects in mouse ES cells. Interestingly, HC2, but not HC1 activates YAP nuclear localization, TEAD-dependent transcription and Nanog promoter activity. HC1 and HC2 share only 39% identity, despite the fact that they both have the same structural domains, a vault protein IαI domain, and a von Willebrand factor type A domain. Most studies so far have used the entire IαI molecule, making it difficult to distinguish the effect of the different HCs. However, a few studies have demonstrated different effects of HC1 and HC2. Tamra *et al.* showed that HC2 acts as a natural inhibitor of brain tumor invasion, and that addition of conditioned medium from cells expressing HC2 or forced expression of HC2 in the glioma cell line inhibits invasion and proliferation *in vitro* by inhibiting the PI3K/Akt pathway (33). Another study by Sanggaard *et al.* suggests that HC2 is necessary for the TSG-6-mediated transfer to take place, possibly explaining both the inability of HC1 alone to induce TEAD family and the need for higher concentrations of HC2 to induce a robust effect on TEAD activity (45). Interestingly, Zhang *et al.* studied the presence of HCs coupled to hyaluronan in amniotic membrane, and found that whereas the amniotic membrane produces HC1, HC2, and HC3, only HC1·HA complex was found in physiological amniotic membrane extracts (44), which could account for the anti-inflammatory, anti-scarring, and anti-angiogenic effects that this particular HC·HA complex yields (46).

Up until now, IαI has mostly been studied as a structural component of the ECM and only a limited number of recent studies have shown the ability of IαI to promote specific cell responses. Moreover, most of these studies have focused on other components in the complex, such as hyaluronan, fibronectin, and vitronectin (31, 34, 42, 46, 47). Therefore the mechanism through which IαI can signal to the cells is still not known. It could be related to its binder hyaluronan, as there are several articles that have shown CD44 binding to hyaluronan to be able to induce cell signaling, for example through Akt phosphorylation (48). There is also the possibility of an integrin-mediated signaling, using IαI's ability to bind to vitronectin (34), as integrin-mediated attachment has been also described to induce cell signaling such as PI3K/Akt phosphorylation (49). Moreover, recent articles have linked YAP to cellular responses induced by extracellular matrix stiffness, showing that hES cells, for example, have increased YAP nuclear localization when seeded on stiffer gels (50, 51). IαI may therefore not signal to the cells directly, but modify the hyaluronan network, changing the mechanical properties of the surrounding matrix of the cells, which in its turn could result in a cellular response such as YAP nuclear localization.

In conclusion, our results show, for the first time that 1 α I is a component of the ES cell ECM network and has the capacity to activate intracellular signal transduction pathways linked to key regulatory transcription factors, and could therefore play an important role in ES cell maintenance. Since 1 α I is present in all mES and hES cell cultures, either via supplements in the medium or the matrix/feeders the cells grow on, it is intriguing to speculate that 1 α I may be an unacknowledged crucial ECM component making up the stem cell niche and that it may need to be supplemented to the medium in a purified or recombinant form when moving toward completely defined, xeno-free conditions. We are currently investigating the mode of action for 1 α I induced intracellular signaling as well as its biological function in human and mouse ES and iPS cell cultures.

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I α 1 Activates the Yes/YAP/TEAD Pathway in Mouse Embryonic Stem Cells

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