Innate triggering and antiviral effector functions of Activin A

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Abstract

Background: First-line defence against viral infection is contingent upon rapid detection of conserved viral structural and genomic motifs by pattern recognition receptors, followed by activation of the type I IFN response and establishment of an antiviral state. Novel antiviral functions of bone morphogenetic protein and related activin cytokines, acting in conjunction with, and independently of, type I IFN, have recently been described. How these antiviral effects are mediated and triggered by viral infection has not been defined.

Methods: Microarray and RNAseq data from hepatoma-derived cell lines stimulated with Activin A in vitro were interrogated both by pathway analysis and for evidence of IFN-stimulated gene induction. Liver tissue obtained from patients with chronic HCV were examined by real-time quantitative polymerase chain reaction (RT-qPCR) for evidence of Activin A induction. Activin expression by peripheral blood mononuclear cells exposed to nucleic acid analogues was quantified by RT-qPCR, whereas induction dynamics in acute infection was investigated in in vitro Sendai virus infection and a murine influenza A.

Results: Transcriptomic analyses delineated strikingly congruent patterns of gene regulation in hepatocytes stimulated with recombinant Activin A and IFNα in vitro. Activin A mRNA, encoded by INHBA, is induced upon activation of RIG-I, MDA5 and TLR7/8 viral nucleic acid sensors in vitro, across multiple cell lines and in human peripheral blood mononuclear cells. In vivo, imurine influenza A also upregulated Inhba mRNA in the lung; this local upregulation of Inhba is retained in MAVS knockout mice, indicating roles for non-RIG-I-like receptors in its induction. Activin induction and signalling were also detectable in patients with chronic viral hepatitis.

Conclusions: These data suggest Activin A is triggered in parallel with type I IFN responses and can trigger related antiviral effector functions, with implications for the development of targeted antiviral
therapies and revealing novel facets of Activin biology.

**Keywords**
Innate immunity, Activin, TGFbeta cytokines, BMP, pattern recognition receptor, viral infection

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Introduction
First identified as a regulator of ovarian folliculogenesis, activin A, a heterodimeric assembly of inhibin β subunits (encoded by the INHBA gene), is implicated in diverse biological processes. In terms of innate immune functionality, activin A is induced in peripheral blood mononuclear cells exposed to pro-inflammatory stimuli including TNFα, GM-CSF and IFNγ; neutrophil granules are also rich sources of pre-formed activin A protein. In vivo, the induction kinetics of activin A in response to systemic inflammatory stimuli have been examined following intravenous LPS exposure: serum activin A protein increases rapidly during endotoxaemic shock in mice, with administration of its soluble antagonist follistatin (FST) sufficient to reduce subsequent mortality. Clinically, in H1N1 influenza-infected patients admitted to intensive care, elevated serum levels of both activin A and FST were detected, and correlated with degree of respiratory distress.

Recent observations have shown an antiviral function of the TGFβ-superfamily cytokines the bone morphogenetic proteins (BMP) and the activins, both transduced via SMAD transcription factors, a phenomenon elucidated via examination of the mutually antagonistic interactions between Hepatitis C Virus (HCV) and the BMP/SMAD signalling axis. Activin A exerts dose-dependent antiviral effects against an in vitro HCV genomic replicon. Additionally, in vitro antiviral functions of activin A against Zika virus, a flavivirus akin to HCV, and also against Hepatitis B Virus (HBV), a structurally distinct hepadnavirus have been reported.

While activin proteins can mediate antiviral effects against multiple viruses in vitro, upregulation of activins in response to viral infection, akin to the rapid induction of type I IFN, has not previously been described. Three classes of pattern recognition receptor (PRR) sense viral nucleic acids: RIG-I-like receptors (RLR); Toll-like receptors (TLR); and the cGAS-STING axis. RLR activation drives the oligomerization of the RIG-I paralogue demonstrating length-dependent activation by non-self RNA. The RLR family also includes MDA5, a RIG-I paralogue demonstrating length-dependent activation by dsRNA. RLR activation drives the oligomerization of the adaptor protein MAVS, the essential factor for downstream type I IFN induction.

In humans, four endosomal TLRs are sensitive to non-self nucleic acids: TLR3, TLR7, TLR8 and TLR9. TLRs comprise an ectodomain conferring ligand specificity; a transmembrane region; and a cytosolic Toll/IL-1 receptor that ultimately activates IRAK, IKK and TBK1 kinases.

In this study, we first used microarray analysis and RNA sequencing to analyse the effects of activin A upon hepatocytes at the transcriptional level and examine its intersection with the type I IFN axis. We next addressed whether activin A transcription is induced upon both PRR activation and viral infection, both in vitro and in vivo, in addition to in part delineating the mechanistic basis for this phenomenon. Overall these data indicate that Activin plays a role in virus infections as part of the innate response.

Methods
Transcriptomics pipeline: extraction and sample preparation
Total RNA was prepared after stimulation or that of untreated controls with the RNeasy Minu' Plus Micro Kit (Qiagen, UK cat# 74106/ 74034). Stimulated cells were lysed in buffer RLT (Qiagen) and homogenized with a QiAshredder (Qiagen). RNA was quantified spectro-photometrically and the quality of RNA preparations for Illumina microarray analysis was checked with an Agilent Technologies 2100 Bio-analyzer.

Two-step reverse transcription was performed on the RNA using AppScript cDNA synthesis kit (Appleton Woods) and quantitative PCR performed using the Roche Light Cycler 480. Approximately 500ng of total RNA from cells was reverse transcribed using the AppScript cDNA synthesis kit. Briefly, total RNA was mixed with kit components App RTase and App cDNA mix to a final volume of 20microlitrel. The mix was then incubated at 42°C for 30 mins. At the end of the reaction RTase was inactivated by heating the reaction to 85°C for 10 mins.

For RT-qPCR validation, 5ul of a 1/20th dilution of resultant cDNA was used as a template for qPCR using the Roche light cycler 480 to detect expression of selected ISGs. Primers were designed using the Roche Universal Probe Library system. Relative gene expression was calculated using the comparative cycle threshold method normalized to expression of the house-keeping gene GAPDH and expressed relative to a mock treated sample.

Transcriptomics pipeline: microarray analysis
500ng of total RNA was used for microarray analysis and was performed by the Oxford Genomic Centre at the Wellcome Trust Centre for Human Genetics, University of Oxford. The quality of the RNA was to have a RNA integrity number (RIN) greater than 7 and 28S/18S ratio of greater than 1.6.

A whole genome gene expression analysis was performed utilizing the Human HT12v4.0 Expression Beadchip. The RNA was converted to biotin labelled cRNA which was then hybridised to the chip. The hybridised chip was then scanned using Illumina’s iScan scanner.

The gene expression profile was then created using illumina’s GenomeStudio software. Data submitted to GEO (accession number GSE190277).

Transcriptomics pipeline: RNA sequencing analysis
The mRNA fraction was selected from the total RNA provided before conversion to cDNA. dUTP was incorporated during second Strand cDNA synthesis. The cDNA was end-repaired, A-tailed and adapter-ligated. Before amplification the samples were uridine digested. The prepared libraries were...
then size selected, multiplexed and QC’ed before paired end sequencing over one rapid run. The data was then aligned to the reference and quality checked.

Cell lines
OR6 cells (kindly from Prof Raymond Chung [MGH, MA, USA]) with permission of Prof Kato and Dr Ikeda [Okayama University, JP]) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Foetal Calf Serum (FCS) (PAA, AT or Lonza, CH), 100U/mL penicillin (Sigma-Aldrich, USA), and 0.1mg/mL streptomycin (Sigma-Aldrich) and 2mM L-glutamine (Sigma-Aldrich); hereafter referred to as “D10”. OR6 cells are stably transfected with a full-length HCV genomic replicon in tandem with a Renilla luciferase reporter and neomycin resistance cassette. OR6 cells were under negative selection with G418 100 μg/mL (Sigma-Aldrich).

Huh7 (ATCC, USA) and Huh7.5.1 (kindly from Prof Raymond Chung [MGH, MA, USA]) were maintained in D10.

HepaRG were cultured at 37°C, 5% CO2 in Williams’ E medium (with Glutamine) (Gibco-Invitrogen) supplemented with glutamine (2 mM), penicillin/streptomycin (50 U/mL), gentamyacin (20 μg/mL), insulin bovine (5 μg/mL, Roche-Boehringer, Germany), hydrocortisone hemisuccinate (7×10−5 M, Roche-Boehringer-Manheim) and FCS (10% selected, non-decomplemented, Fetaclone II-Hyclone-PERBIO France). The cells were allowed to differentiate in the presence of 2% (v/v) DMSO to the medium.

A549.gfp cells (a kind gift from Prof Richard Randall, University of St Andrews, UK) were maintained in D10.

Patients samples
HCV patient samples were obtained from patients enrolled at the JR Hospital, Oxford and samples stored under protocol 16/YH/0247. Liver biopsy samples were collected prior to commencement of anti-viral therapy and were graded and staged using ISHAK scoring at S. Bortolo Hospital Vicenza, Italy. RNA extraction, reverse transcription and qRT-PCR was performed as described below. Ethical approval for use of the biopsy samples was obtained from the relevant local ethics committees. Informed written consent was obtained from all patients involved.

Peripheral blood mononuclear cells extraction
Whole blood was procured from nine healthy volunteer donors (adult, predominantly female, Caucasian, South Asian and Middle Eastern ethnic groups) in accordance with local ethical policy and practices, as described previously[154]. Blood samples were heparin-treated before immediate layering onto a Ficoll-Paque Plus™ (GE Healthcare Life Sciences, USA) density centrifugation gradient in a 1:1 volume ratio. Samples were centrifuged and theuffy coat layer aspirated with a pipette. Buffy coat cells were washed three times in 2.5 mM EDTA-PBS at 4°C before dilution and maintenance in RPMI-1640 supplemented with 10% FCS (PAA, AT or Lonza, CH) 2 mM glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin (all Sigma-Aldrich).

Enrichment of CD14 monocytes
CD14 positive cells were isolated from the PBMCs using the EasySep™ Human CD14 Positive Selection Kit by STEMCELL Technologies™ (Cat# 18058).

Synthetic nucleic acids and base analogues
High molecular weight poly(LC) [cat. I.D. tlrl-pic], poly(dA: dT) [cat. I.D. tlrl-patn], E. coli K12 dsDNA [cat. I.D. tlrl-ecdna], ODN 2216 [cat. I.D. tlrl-2216], R837 [cat. I.D. tlrl-imqs], R848 [cat. I.D. tlrl-r848] and ssRNA40/LyoVec™ [cat. I.D. tlrl-1ra40] were purchased from InvivoGen (FR). IVT-RNA transcribed by the T7 RNA polymerase and corresponding to nucleotides 1–99 of the neomycin phosphotransferase gene was synthesized as described in 15 (kindly from Prof Jan Rehwinkel [University of Oxford, UK]).

Transfection and stimulation of unfractionated PBMC and CD14+ monocytes
Human PBMC were extracted from healthy donors as described in section 2.1.6 and plated at 1x10^5 cells/well of a 24-well plate. PBMC were transfected with IVT-RNA and poly(I:C) using Lipofectamine® LTX with PLUS™ (Life Technologies). R848, R848, ODN 2216 and ssRNA40 were added to the culture medium in the absence of transfection reagent to the end-point concentrations specified.

CD14+ cells were plated at at 1x10^6 in 300 μl R10 medium per well and stimulated with HBV inoculum (final MOI = 0.5–1), HCV inoculum (final MOI = 0.5–1). Cells were incubated at 37°C, 5% CO2 for 16 hours. Cells and supernatants were harvested and frozen at -20°C for further analysis.

In vitro Sendai virus (SeV) Infection
Cantell strain Sendai Virus [cat. I.D. ATCC® VR-907™] was purchased from ATCC. A549, Huh7 or Huh7.5 cells were plated at 5x10^4 cells/well in 12-well plates and incubated at 37°C for 24h. Immediately prior to infection, D10 was aspirated and the monolayers washed twice with PBS, before addition of SeV at MOI 0.5, MOI 1 and MOI 5 in 500uL serum-free DMEM. Cells were incubated with SeV for 2h at 37°C, before removal of the virus, two PBS wash/aspirations and replenishment with D10.

In vitro dengue virus (DENV) infection
Huh7.5 cells were plated at 3x10^4 cells/well in 12-well plates and allowed to adhere. Cells were incubated with DENV2 (strain 16681) in serum-free DMEM for 2 h at room temperature with gentle agitation, followed by removal of virus, washing with PBS and incubation at 37°C for 48h in D10.

In vivo influenza A virus (FLUAV) infection
All efforts were made to ameliorate any suffering of animals used within this study through minimization of numbers involved and optimal housing conditions. Experiments were
performed in accordance with local ethics policies and permissions. For Figure 4A–E, six-week-old female C57BL/6 mice were anaesthetized and infected intranasally (i.n.) with 3.5 haemagglutinating units FLUA V PR/8/34 (H1N1), kindly provided by Prof John Skehel (NIMR, Mill Hill, UK). A randomization strategy was not used to allocate mice to infection versus control groups. Control mice were administered PBS i.n. Mice were sacrificed at 72 h.p.i. by asphyxiation. Whole lungs were immediately lysed in RLT buffer (Qiagen) with 10 μL mL−1 β-mercaptoethanol and mechanically disrupted with a TissueRuptor (Qiagen), before RNA extraction, cDNA synthesis and RT-qPCR quantification of gene expression as described in section 2.2. Liver explants (~2 mm³) were preserved in RNAlater (Qiagen), before mechanical lysis with a TissueRuptor and RNA extraction, cDNA synthesis and RT-qPCR quantification of gene expression.

For Figure 4F–G, six-week-old female wildtype C57BL/6 mice were infected i.n. with increasing titres of wildtype FLUA V PR/8/34 from 4×10⁴ to 5×10⁶ pfu. Control mice were administered DMEM i.n. Mice were sacrificed at 48 h.p.i. by asphyxiation, followed by RNA extraction, cDNA synthesis and RT-qPCR quantification of gene expression. After sacrifice, whole lungs were removed and snap-frozen in liquid N₂. 1 mL Tri Reagent (Sigma-Aldrich) was added to each lung, before lysis with glass beads [cat. I.D. G8772] (Sigma-Aldrich) in a FastPrep Cell Disruptor. RNA was extracted from the lungs with lysates with phenol-chloroform followed by isopropanol precipitation Sacchi. RNA isolates were further purified, and depleted for genomic DNA, by secondary extraction using the RNeasy Plus kit (Qiagen). cDNA synthesis and RT-qPCR analysis of gene expression were conducted as described previously.

For Figure 4H–I, six-week-old female C57BL/6 mice, both wildtype and MAVS KO¹⁷ were infected i.n. with 5×10⁴ pfu wildtype FLUA V PR/8/34. Mice were sacrificed at 48 h.p.i. by asphyxiation. Control mice were administered DMEM i.n. Whole lungs were harvested and RNA extracted as per the preceding instructions, followed by cDNA synthesis and RT-qPCR quantification of gene expression. Animal experimentation was in line with requirements stipulated in the ARRIVE guideline checklist.

RNA isolation and quantification
Unless otherwise specified, cell lysates were homogenized with a QIAshredder column (Qiagen) and RNA extracted with the RNEasy Mini Kit (Qiagen). RNA concentration was determined with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, MA, USA) at 260 nm. The quality of RNA preparations for Illumina microarray analysis and RNA sequencing analysis was checked with an Agilent Technologies 2100 Bio Analyser.

cDNA synthesis and RT-qPCR analysis
cDNA was reverse-transcribed from template RNA either using a two-step reverse transcription using AppScript cDNA synthesis kit (Appleton Woods, UK; catalogue number ARP602) or using the High Capacity RNA-to-cDNA kit (Applied Biosystems, USA; catalogue number 4387406).

All RT-qPCR reactions were either performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, USA). For TaqMan™ quantification, gene expression was assessed with inventoried TaqMan™ Gene Expression Assays (Applied Biosystems, MA, USA) diluted in TaqMan™ Gene Expression Master Mix (Applied Biosystems, USA). TaqMan™ inventoried assays used: GAPDH (glyceraldehydef 3-phosphate dehydrogenase) Hs99999905_m1; INHBA (inhibin beta A) Hs01081598_m1; MX1 (myxovirus resistance 1, mouse, homolog of) Hs00895598_m1; IFI6 (interferon-alpha-inducible-protein 6) Hs00242571_m1; IFNAR2 (Interferon alpha, beta and omega, receptor 2) Hs01022061_m1; JAK1 (Janus kinase 1) Hs01026983_m1; STAT2 (Signal transducer and activator of transcription 2), Hs01013123_m1; Hprt (hypoxanthine-guanosine phosphoribosyltransferase) Mm01545399_m1; Inhba (inhibin beta A) Mm00434339_m1; Isg15 (ubiquitin-like modifier ISG15) Mm01705338_s1; Trim14 (tripartite motif-containing protein 14) Mm01352552_m1 or quantitative realtime PCR analysis was performed using the Roche Light Cycler 480 instrument using AppProbe reagents (Appleton Woods). Primers were designed using the Roche Universal Probe library system. Relative gene expression was calculated using the comparative cycle threshold method¹² normalised to expression of the housekeeping gene GAPDH and expressed relative to a mock treated sample. The primer list used in Figure 1 D and E, Figure 2 A and B, Figure 4 A are indicated in Supplementary Table 1 (Extended data)²⁷.

Plasma cytokine and Activin A quantification
Concentrations of interferon alpha 2 in cell culture supernatants was measured using custom multiplex immunoassay kits (GeniePlex, Ireland).

Concentration of Activin A in human and mouse serum samples were performed using sandwich ELISA- Activin A immunoassay kit from R&D system (Cat DAC008).

Bioinformatic and statistical analysis
The Illumina bead chip output files were processed and analysed using the R statistical software (v 2.11)¹⁹ and statistical testing was performed using the Linear Models for Microarray Analysis (limma) package and DESeq²⁰,²¹. Differential gene expression between the experimental groups was assessed by generating relevant contrasts corresponding to the possible cell type comparisons. Raw p-values were corrected for multiple testing using the false discovery rate controlling procedure of Benjamini and Hochberg¹³; adjusted p-values <0.01 were considered significant. Gene set enrichment analysis methods (GSEA) are as described²²,²². Pathway representation was performed using the MetaCore pathway analysis software from Clarivate Analytics. Unless otherwise specified, data transformation and analysis was performed with Microsoft Excel (Microsoft Inc., USA). Statistical analysis and data presentation were performed with GraphPad Prism (GraphPad Software
Figure 1. A) Diagram representing experimental cell culture procedure on stimulation of HepaRG cells with Activin A. B) C) Metacore analysis performed on differentially regulated genes identified by microarray analysis of RNA from HepaRG cells stimulated with 10nM activin A over 24 hours. Figure 1B highlights pathways mapped to the 184 significantly upregulated genes; Figure 1C highlights those mapped to the 168 significantly downregulated transcripts. D) Time course over a period of 72 hours of HepaRG cells stimulated with Activin A (10nM), with relative gene expression levels of various ISGs shown compared to unstimulated cells. n=3 independent experiments conducted in triplicate. E) Comparative analysis of gene expression in different hepatocyte cell lines (HuH7, HepaRG, HHL12) at 24 hours following stimulation with Activin A (10nM). n=3, independent experiment conducted in triplicate.
Figure 2. A) Gene Set Enrichment Analysis (GSEA) showing consistency of results between microarray and RNA-sequencing experiments interrogating transcriptomes elicited by treatment of HepaRG hepatoma-derived cells with activin A 10nM over a period of 24 hours. The whole set of up-regulated genes (log FC >0, p value > 0.1, n=2571) between Activin vs Untreated in RNA-seq were found significantly enriched (FDR = 0.04, p << 0.01) in Activin-treated samples derived from the equivalent microarray experiment described in Figure 1A (Activin vs Untreated condition). B) C) GSEA showing enrichment of Interferon gene pathways in transcriptomics from Activin treated samples. Based on 110 Interferon-related signatures selected from MsigDb, two representative Interferon Gene Sets were defined, corresponding to Interferon Signalling (Figure 2B) and Interferon Gamma Signalling. Both of these gene sets were found to be significantly enriched in RNA-derived from HepaRG cells stimulated with activin A 10nM over 24 hours (Figure 2A: FDR = 0.19, p=0.026; Figure 2B: FDR = 0.019, p=0.004 respectively.
Inc., USA). Analysis with R and the ggplot2 package provides a free-to-use alternative tool for these analyses.

**Results**

**Activin A stimulation upregulates immune pathways in HepaRG cells**

As shown in Figure 1A, in order to better understand the effect of Activin A on hepatocytes, we used microarray analysis of the RNA isolated from HepaRG cells activated with 10nM Activin A for 24 hrs. Differential analysis of the expression data show that 184 genes were upregulated and 168 number of genes were down-regulated with a minimum of 1 log fold change. Table 1 shows the top 40 list of differentially regulated genes (limma, paired t test p<0.001 lfc>1). Metacore analysis (Clarivate Analytics) of the differentially regulated genes show that genes in Type 1 alpha/beta signalling pathway were predominantly upregulated, followed by MHC Class I presentation and Response to RNA viral infection (Figure 1B). However, Activin A seems to be downregulating genes in the cell cycle pathway (Figure 1C).

**Activin A stimulates ISGs in hepatocyte cell lines**

Intrigued by the ability of Activin A to up-regulate ISG in the absence of Interferons, we looked to address whether Activin A increases ISG levels by regulating IFN expression in the HepaRG hepatocyte cell line over a 72 hr period (Figure 1D). RT-qPCR analysis validated the increase in ISG expression observed in microarray and RNA sequencing. HepaRG cells stimulated with Activin A showed an increase in expression of various ISGs relative to un-stimulated cells (Figure 1D). However, when tested on other cell lines, namely Huh7 hepatoma-derived cells and the immortalised transformed hepatocyte cell line HHL12, we observed that the pattern on gene expression over time is not consistent and individual cell lines behave differently to Activin A in terms of ISG expression (Figure 1E). The primer pairs used for the qPCR are detailed in Supplementary Table 1 (Extended data).

**RNA-sequencing confirms results of microarray data analysis on hepatocytes stimulated with Activin A**

We corroborated our data obtained by microarray analysis by performing a new RNA sequencing experiment on a separate experiment with hepatocytes stimulated with Activin A. We then performed a Gene set enrichment analysis (GSEA) comparing both experiments. GSEA shows high correlation in gene expression by both methods of analysis. The entire set of Activin vs Untreated up-regulated genes detected in the RNA-Seq experiment (n=2571, data processed with DESeq2 package) were found highly enriched compared to the microarray data (Figure 2a).

To show that ISG were upregulated in response to activin induction, we performed a GSEA using a large set of Interferon signatures publicly available in MsigDB database. We selected 110 gene sets querying all curated databases (e.g Reactome, GO, etc.) and reported gene expression data from immunology and cancer studies (e.g. Up-regulated genes on cells stimulated with IFNα) in MsigDB. We found that 88 of all 110 gene sets were up-regulated in Activin treated samples, of which 24 gene sets were significantly enriched at FDR < 25%, 10 gene sets were at nominal p-value < 0.01, and 19 gene sets were significant at nominal p-value < 0.05. The remaining 22/110 gene sets were enriched in Untreated samples without any statistical significance. Figure 2b and Figure 2c shows two examples of public IFN signatures enriched in up-regulated genes in our Activin vs Untreated samples (in RNA-Seq data after voom transformation in limma R package).

**Activity of Activin A is independent of IFNa**

We have previously shown that recombinant Activin A protein exerts a dose dependent antiviral effect. We first explored the transcriptional pathways and show that Activin A is able to induce ISG signalling in the presence of B18R, a type I IFN binding protein derived from Western Reserve Strain Vaccinia virus, in Huh7 cells (Figure 3A). However, B18R is able to inhibit RNA expression of ISG in response to IFNα (Figure 3B). In addition, supernatants of hepatocyte (Huh7) cells stimulated with Activin A did not show the presence of IFNα2 (Figure 3C) supporting the view that Activin A acts independently of type I IFN. Additionally it was observed that the antiviral functions of Activin A does not require signalling via the type I IFN receptor (IFNAR), being unaffected by ruxolitinib, a pharmacological inhibitor of the JAK1 kinase downstream of IFNAR [see Supplementary Figure 1A–C, Extended data].

**Evidence of in vivo expression of Activin A in viral infection**

To further examine whether Activin A is expressed during the course of viral infection in vivo, we performed PCR to detect Activin A mRNA expression levels in livers and ELISA to detect Activin A protein in serum of patients with HCV infection. Figure 4A shows that Activin A levels in inflamed liver samples from HCV patients were significantly increased. Significant increases in Activin A protein levels in the non-responders to IFN therapy were observed compared to controls (Figure 4B). To assess whether Activin A could also be induced in an acute viral infection we inoculated mice with MCMV intravenously. In this model Activin A was detected at 3 days post-infection (Figure 4C). These data indicate that acute and chronic viral infections both lead to induction of Activin A in vivo.

**Activin A is induced by PRR activation in human peripheral blood mononuclear cells**

In order to explore the signalling mechanisms responsible for Activin A induction by infectious stimuli, freshly-isolated human peripheral blood mononuclear cells, which express a broad repertoire of cytosolic and endosomal viral sensors, were transfected transfection with IVT-RNA or poly(I:C). These stimuli, detected by RIG-I and MDA5 respectively, elicited a statistically significant upregulation of INHBA mRNA (Figure 5A), mirrored by significant transcriptional induction of MX1 and IFI6, a pleiotropic antiviral effector (Figure 5B, 5C).

Incubation with the synthetic guanidine base analogues R837 and R848 (TLR7 and TLR7/8 stimuli respectively) at 1 μg/mL resulted in approximately 15-fold and 50-fold increases in INHBA expression respectively (Figure 5D), implying a role
Table 1. Summary of the top forty differentially regulated genes identified via microarray analysis of RNA extracted from HepaRG cells exposed to activin A for 24 hours (obtained via the limma package; paired t-test p<0.001, log Fold Change (lfc) >1).

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**Figure 3.** A) Gene Set Enrichment Analysis performed on two sets of genes obtained by RNA sequencing and microarray analysis. Results show high correlation in gene expression by both methods of analysis. Metacore analysis showing genes from significant canonical pathways in hepatocytes stimulated with Activin A, BMP6 and IFNa respectively. Metacore analysis showing genes from significant process network in hepatocytes stimulated with Activin A, BMP6 and IFNa respectively.
for TLR7 in INHBA induction. This was parallel by robust induction of MX1, encoding the broadly antiviral GTPase MX1 (Figure 5E). Statistically significant INHBA upregulation following incubation with ssRNA40 at 1 μg/mL and 5 μg/mL (Figure 5F), with attendant induction of IFI6 (Figure 5G), suggesting a role for TLR8 in INHBA induction in vitro.

Recapitulating these observations with live viral inocula, incubation of human CD14+ monocytes with HBV and HCV (multiplicity of infection 0.5–1.0) for 16 hours resulted in significant induction of INHBA mRNA and detectable activin A protein in the culture supernatant (Figure 5H).

INHBA mRNA is upregulated by ssRNA virus infection in vitro

Having clarified the similarity of activin A and type I IFN in terms of patterns of antiviral gene regulation in clinical infection models and also looked at its induction by synthetic immune stimuli, our attention turned to the possibility that activin itself is induced by viral infection. In RIG-I/TLR3 replete lung adenocarcinoma-derived A549.gfp cells, infection with the ssRNA paramyxovirus Sendai virus (SeV) results in a statistically significant, dose-dependent induction of INHBA mRNA (Figure 6A), paralleled by a strong induction of MX1 (Figure 6B). SeV encodes a 546nt immunogenic motif within its 5’-UTR and intact RIG-I, but not MDA5, signalling is necessary for IRF3 phosphorylation and subsequent ISG induction in vitro.30

To further clarify the PRR requirements for INHBA induction by SeV, Huh7 and Huh7.5 hepatoma-derived cells were infected with a titration of SeV, and expression of INHBA, MX1 and genomic SeV quantified by RT-qPCR. The RIG-I isoform expressed by Huh7.5 encodes an N-terminal point mutation that uncouples viral sensing from downstream signalling30. Huh7 cells and their derivatives are therefore deficient for TLR3 expression.

In RIG-I replete Huh7 cells, SeV infection elicits titre-dependent INHBA upregulation [Figure 6C], paralleled by strong induction of the IFN-dependent target gene MX1 at 24 hours post infection (Figure 6D). In Huh7.5.1 cells, however, neither INHBA nor MX1 message levels were altered by SeV infection. Baseline expression of both INHBA and MX1 is approximately 10-fold higher in uninfected Huh7.5.1 cells versus Huh7. SeV genomic RNA levels were comparable in both cell lines, although the signal in the uninfected conditions implies low-level off-target amplification (Figure 6E).

Moving on to consider in vitro flavivirus infection, type I IFN induction secondary to dengue virus (DENV) is reportedly contingent upon MAVS-dependent RLR signalling31. MDA5 is likely to represent the principal sensor of DENV in Huh7.5.1 cells, being deficient in functional RIG-I and TLR329,30. At 48 hours post infection, DENV infection in Huh7.5.1 cells results in statistically significant upregulation of mRNA encoding INHBA (Figure 6F), accompanied by a significant upregulation of MX1 (Figure 6G).

We quantified INHBA expression in Huh7 cells incubated with recombinant IFNα-2a, and established that INHBA mRNA is not induced by recombinant type I IFN in this cell line (see Supplementary figure 1A, Extended data38). Akin to the antiviral enhancement of type I IFN by BMP6, co-incubation of OR6 HCV genomic replicon cells with recombinant activin A synergistically enhances the antiviral effect of IFNα-2a, hinting at possible functional ramifications of viral INHBA induction (see Supplementary figure 1 B–D, Extended data38).
Influenza A infection induces *Inhba* expression in the lungs of C57BL/6 mice

Moving to *in vivo* infection models, we observed that *Inhba* expression is significantly upregulated in the lungs of C57BL/6 mice infected with the influenza A [FLUA V] strain PR/8/34 for 72 hours compared to uninfected controls (Figure 7A). This induction correlates tightly with that of *Isg15*, encoding a pleiotropic, IFN-stimulated antiviral effector\(^2\) (R\(^2\) = 0.9271, p=0.0020) (Figure 7B–7C). In the liver, however, *Inhba* expression is significantly suppressed by FLUA V (Figure 7D), despite no alteration in *Isg15* expression (Figure 7E). Suppression of hepatic *Inhba* expression in the presence of a systemic inflammatory response has been previously reported, albeit in the context of LPS administration\(^3,4\), not viral infection.
Upon intranasal infection with titrating doses of FLUA V, *Inhba* is dose-dependently upregulated at 48 hours post infection, reaching maximal a three-fold increase with the $5 \times 10^4$ plaque forming unit (pfu) inoculum (Figure 7F).

To gain a mechanistic insight into the PRR subsets responsible for *Inhba* induction during intranasal FLUA V infection, we compared *Inhba* expression in lung lysates from both wildtype (WT) and Mavs knockout (KO) mice. These animals cannot transduce RLR signalling. In wildtype and Mavs KO mice, *Inhba* expression was significantly increased by intranasal administration of $5 \times 10^4$ pfu FLUA V at 48 hours post-infection, albeit by a smaller fold change in the transgenic animals (Figure 7G). Similarly, the magnitude of both *Inhba* and *Isg15* induction was lower in Mavs KO compared to WT mice, although this difference did not attain statistical significance (Figure 7H). These data suggest a function of Mavs-independent pattern recognition in *Inhba* induction by FLUA V.

**Discussion**

An antiviral function of the BMP/activin-SMAD signalling axis has been described in the context of viral infection. Here, we
Figure 7. A) B) C) Inhba and Isg15 mRNA are upregulated in the lungs of FLUAV PR/8 infected mice at 72 h.p.i. The degree of their respective induction is strongly positively correlated (R² = 0.9271, p = 0.0020). n = 5 uninfected and n = 6 infected mice; mean ± SEM; analysis by unpaired two-tailed t-test (A, B) and linear regression (C). D) E) At 72 h.p.i. in the liver, Inhba message is significantly suppressed in the infected mice, whereas hepatic levels of Isg15 message do not differ between groups. n = 8 mice per group; mean ± SEM; analysis by unpaired two-tailed t-test. F) Lung Inhba expression is dose-dependently induced by FLUAV infection at 48 h.p.i. n = 3 mice per group; mean ± SEM; analysis by ordinary one-way ANOVA and Bonferroni’s post-test with respect to uninfected mice. G) H) mRNA encoding both Inhba and Isg15 is significantly upregulated in the lungs of both WT and Mavs KO mice at 48 h.p.i. with FLUAV; no significant difference in the magnitude of induction is evident between groups.
show that gene expression signatures associated with activin stimulation are induced in parallel to those downstream of type I IFN in a clinically important viral disease, namely chronic HCV infection. Additionally, we provide evidence for induction of activin A, encoded by the INHBA gene, in multiple models of viral infection, both in vitro and in vivo. Limitations of our study include that no definite correlation of mRNA induction with activin protein production our in vitro and in vivo models of infection has been established; future studies must investigate whether message level induction extends to protein synthesis and extracellular release.

The transcriptomic analyses described here suggest that stimulation of antiviral effector genes classically regarded as “interferon-stimulated” may not be an exclusive feature of IFN, as both BMP6 and activin A are able to upregulate ISG in the absence of exogenous IFNα and ISG induction occurs as a summation of diverse unrelated signalling events. Together with the observed antiviral properties of recombinant activin A, we propose that its transcriptional induction downstream of viral sensing may represent a hitherto undescribed antiviral feedback response, initiated upon detection of infection and acting to curtail viral replication.

Wu and colleagues report that in the resting state, murine alveolar and bronchial macrophages stain positively for activin A protein; during LPS endotoxaemia, activin A positive neutrophils aggregate in the perialveolar spaces. This reported accumulation of activin A-rich neutrophils in the lung correlated with reduced numbers in the bone marrow, consistent with migration into the systemic circulation. It was posited that this activin A induction occurred at the post-transcriptional level, being ablated by cyclohexamide but unaffected by the transcription initiation inhibitor actinomycin D. In contrast, our data obtained with FLUAV demonstrate transcriptional upregulation of Inhba, with respiratory epithelia and alveolar macrophages being equally plausible candidate sources.

In vivo, Inhba mRNA is upregulated in the lungs of mice following intranasal FLUAV infection. This induction is not abrogated upon Mavs knockout, implying a contribution of Mavs-independent viral sensing. Data from human PBMC in vitro indicate that TLR7/8 activation – that is, independent of MAVS – is sufficient for INHBA upregulation. As such, definitive characterization of Inhba induction in vivo will require examination of mice deficient in Tbk1, the point of RLR and TLR convergence upon IRF3.

Our observations of INHBA induction in experimental infection systems correspond with clinical reports of elevated serum activin A protein in both acute influenza A infection, where it correlates with the degree of respiratory distress, and in chronic HCVs. Interpretation of these patient studies is complicated by the well-established roles of activin A and TGFβ signalling in tissue fibrosis; it is impossible to dissect whether elevated activin A occurs immediately downstream of viral sensing or as a mediator of fibrotic immune pathology.

Given the described antiviral properties of exogenous activin proteins and manipulation of the activin/SMAD signalling axis, these data nevertheless provide an essential first step in the clarification of activin A’s endogenous role in the innate response to viral infection.

**Data availability**

**Underlying data**


This project contains the following underlying data:
- Figure 5A, 5B, 5C, 5D, 5E, 5F,5G.csv
- Figure 6A, 6B, 6C, 6D, 6E, 6F, 6G.csv
- Figure 7A, 7B, 7C, 7D, 7E, 7F, 7G, 7H.csv


This project contains the following underlying data:
- Figure 1D, 1E.xlsx
- Figure 3A, 3B, 3C.xlsx
- Figure 4A, Figure 4.xlsx
- Figure 5H, Figure 5I.xlsx

**Extended data**

Supplementary data table 1 with the DOI: https://doi.org/10.6084/m9.figshare.20439312.v1.


This project contains the following extended data:
- Supplementary Figures 1A, 1B, 1C, 1D.csv

**Reporting guidelines**


Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

**Microarray and RNAseq data**


**Acknowledgements**

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An earlier version of this article can be found on bioRxiv (doi: https://doi.org/10.1101/2021.03.23.436626).


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This study investigates the protein Activin A that has diverse biological functions including potential immunological functions. This study investigates the possible antiviral functionality of Activin A, firstly examining Activin A-induced gene expression prior to investigating pattern recognition receptor induction of Activin A expression.

This is an original study that identifies Activin A induces IFN-associated gene signatures in vitro. The study then demonstrates that Activin A gene expression is IFN-independent, highlighting that Activin A may represent an antiviral protein that exerts antiviral functionality independently of IFN. The authors then show that Activin A expression is induced by RNA viruses and associated pattern recognition sensors in vitro and in vivo. Clinical data suggesting the relevance of Activin A in human viral infections are shown. The main study limitation, namely that Activin A mRNA rather than protein was used as a readout for expression was discussed by the authors.

Overall, this study is a convincing, robust and interesting addition to the literature, and gene expression data represent a useful resource for those studying this immunological protein.

Is the work clearly and accurately presented and does it cite the current literature? Yes

Is the study design appropriate and is the work technically sound? Yes

Are sufficient details of methods and analysis provided to allow replication by others? Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Immunology, viruses, innate immunity, antiviral immunity

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.