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Supplementary Information for

Photothermal Modulation of Human Stem Cells Using Light-responsive 2D Nanomaterials

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Footnote

Author contributions: J.K.C., K.A.S., I.S. and A.K.G. conceptualize the project. J.K.C., K.A.S., M.K.J., A.R., and G.L. performed the experiments. I.S. analyzed and interpreted RNA-seq data. J.K.C., K.A.S., I.S. and A.K.G. prepared figures and wrote the manuscript. All authors have edited the manuscript and given approval to the final version.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE141456).

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Materials and Methods

MoS_2 Synthesis. The bulk MoS_2 was chemically exfoliated following previously reported protocols.(1-3) in a nitrogen filled glove-box, 200 mg of bulk MoS_2 powder (Alfa Aesar) in single-neck 100 mL round-bottom flask was degassed for a couple of hours before adding 3 mL *n*-butyllithium (1.6 M in hexane, Sigma USA). The solution was then refluxed at 70 ˚C for 48h with continuous stirring. The reaction mixture was then cooled to room temperature and added with 20 mL dried hexane and filtered with nylon paper (pore size ~ 0.45µm). The excess *n*-butyllithium was washed and diluted with anhydrous hexane and safely transferred to a hazard labeled glass jar and sealed. The black precipitate was taken out from glove box and dispersed in 200 mL chilled water in a round-bottom flask and sonicated for an hour in an ice-cold water-bath to assist the exfoliation. To remove partially stacked layers from the suspension, the solution was centrifuged at 2 x 5000 rpm for 10 min per cycle followed by 2 x 8000 rpm for another 10 min per cycle. The dispersion was collected and stored at 4 ˚C for further experiments.

MoS_2 Characterization. Transmission electron microscopy (TEM) was performed on a carbon grid at an accelerating voltage of 200 kV using a JEOL-JEM 2010 (Japan). Physical characterization of chemically exfoliated MoS_2 (MoS_2) was investigated in the presence of physiological proteins *via* introduction of lysozyme or albumin. The zeta potential and hydrodynamic size of MoS_2-albumin solutions were measured with a Zetasizer Nano ZS (Malvern Instrument, U.K.) furnished with a He−Ne laser at 25°C. Changes in protein conformation after adding MoS_2 were evaluated using the ANS assay (8-anilino-1-naphthalenesulfonic acid).(4) Fluorescence was analyzed using a Tecan plate reader.

The crystallographic phase of monolayer MoS_2 sheets together with bulk counterpart was confirmed by X-ray powder diffraction (Bruker D8 advanced) using copper K_α source (wavelength, 1.54 Å). The data were recorded from 5 to 70 degrees and obtained peaks were indexed using JCPDS card No. 73-1508. The characteristic peak (002) was used to calculate crystallite size (*D*) using Scherrer equation given as below:

$$D = \frac{K\lambda}{\beta \cos\theta}$$

Where *K* is shape factor (approx. 0.94), λ is X-ray wavelength, β is line broadening at full width half maxima (FWHM), and θ is the Bragg angle.

The exfoliated monolayers of MoS_2 were imaged with transmission electron microscopy (TEM, JEOL 2010) operated at 200 keV. For sample preparation, aqueous dispersion of MoS_2 samples were drop-casted and air-dried on copper grid (procured from Ted Pella Inc.). The selected area electron diffraction (SAED) were recorded for phase data corroboration obtained from X-ray diffraction. Further to determine the thickness of the monolayer MoS_2 along with the topographical features, height profile imaging of the sample was analyzed using atomic force microscopy (AFM, Bruker Nanoscope) in tapping mode. Samples were typically placed and dried onto silica substrate by spin coating at 1000 rpm.

The crystallographic arrangement of Mo-S atoms from 2H phase to 1T takes place during lithium intercalation process. To confirm this, we carried out Raman spectra analysis with

beam operated at 532 nm green laser (LabRam HR confocal Raman microscope, Horiba Inc.). The Raman shift in two prominent characteristic peaks corresponding to E^1_{2g} and A_{1g} were particularly recorded and analyzed as described in the result section. Further to determine the characteristic absorbance peaks of MoS_2, aqueous suspension of the samples was subjected to UV-visible spectrophotometer (Perkin Elmer Inc.) and the date were recorded from 300 to 800 nm. The presence of three peaks at approx. 470, 622, 668 nm confirmed the formation of dominant 2H phase with partial presence of 1T phase. The corresponding photoluminescence (PL) spectra for the monolayer MoS_2 sample, aqueous dispersions were excited at 470 nm and room-temperature emission spectra were recorded from 550 to 700 nm using Spectrofluorometer (Zeiss Inc.) equipped with xenon arc lamp.

The confirmation of 2H phase together with 1T phase obtained from Raman spectra were further corroborated with X-ray photoelectron spectroscopy (XPS, Omicron Inc.). The binding energies (B.E.) for Mo (*3d*) and S (*2p*) for MoS_2 samples along with that of adventitious carbon (C*1s*) for both bulk and monolayer counterparts were recorded. The raw data were further processed and deconvoluted by CasaXPS multiple peak fit software version 2.3.15 and indexed using standard library.

Stem Cell Culture. Human mesenchymal stem cells (hMSCs) (Institute of Regenerative Medicine (IRM)) were cultured under normal media conditions consisting of α-minimal essential media (alpha-MEM, Hyclone, GE Sciences) with 16.5% fetal bovine serum (Atlanta Biologicals, USA) and 1% penicillin/streptomycin (100 U/100 µg/mL, Gibco). After every 2-3 days, half of culture media was exchanged for fresh media. Cells were passaged with 0.5% trypsin-EDTA upon reaching confluency of ~70% and seeded at ~2500 cells/cm^2. All experiments were completed with cell populations under P5. Leaching studies were completed using both 2D exfoliated and bulk MoS_2 (1 mg/mL) placed within transwell membranes in a 24-well plate. As positive controls, solutions of ammonium molybdate salt in equivalent molar concentrations as the physical particles, which came out to 0.6 mg/mL, were placed onto transwell membranes. Cell health was monitored using an MTT assay (ATCC) at time points of 1 day and 7 days.

Protein Corona Formation: The composition of the protein corona formed around the nanosheet was investigated an SDS page gel electrophoresis. Briefly the nanosheets were incubated with serum supplemented alpha MEM media for 2 hours with and without photothermal stimulation. The samples were the samples were centrifuged at 10,000g and washed 3 times with PBS. The nanosheets were then suspend in Laemmli buffer, followed by incubation 90ºC for 15 minutes and subsequent gel electrophoresis was performed. The Gels was then stained using silver stain.

Cell Morphology. For actin staining, hMSCs were treated with MoS_2 for 24 hours, then fixed with 2.5% glutaraldehyde and permeabilized with 0.1% TritonX-100. Phalloidin stain was then added and samples were incubated for 1 hour at 37 °C. The stain was removed, washed with 1X PBS. Actin stained samples were imaged with a fluorescent inverted microscope. Cell area was quantified using ImageJ analysis software. Scanning electron microscopy of hMSCs treated with MoS_2 on glass cover slips were captured after sample (50 µg/mL) treatment for 1 hour. Cells were washed with PBS to remove weakly bound particles. Subsequently cells were fixed with 2.5% glutaraldehyde for 30

minutes at RT, washed again with PBS, and dehydrated using serial dilutions of ethanol and then by placing the slides in hexamethyldisilazane (HMDS) (James Cook University, Biological SEM sample preparation protocol). SEM images were taken on gold sputter-coated samples (JEOL NeoScope).

Cell Culture Assays. To evaluate changes in cell behavior with NIR exposure, cells were incubated under low intensity NIR LED light for two hours over a three-hour period each day. Specifically, an LED array with a 3.4 W output power was placed 6 cm from the surface of the plate. The power density across the plate was measured to be around 20 mW/cm^2. Following exposure with or without MoS_2 (25 μg/mL), cell behavior was monitored through multiple studies.

For cytotoxicity and proliferation assays hMSCs were seeded in 96 well plates at a seeding density of 5000 cells per well. The cells were exposed to varying concertation of MoS2 nanosheets for pre-defined time (24 hrs for cytotoxicity and 24 ,72 ,96 hrs for proliferation assays, respectively) followed by measurement of metabolic activity using Alamar Blue (Thermo Scientific) as per the manufacturers' protocols.

For cell cycle analysis hMSCs were cultures in 6 well plates. After reaching 50% confluency hMSCs were serum starved (only 1% FBS in media) for 24 hrs to synchronize cell populations, followed by treatment with MoS2 nanosheets (25μg/mL). After 72 hrs of exposure the cells are trypsinized and fixed in ice cold 70% ethanol. The cells are then centrifuged and washed with PBS 3 times, followed by incubation with PI (40 μg/mL) and RNase (100 μg/mL) at 37ºC for 1 hr. Cells were stored at 4ºC till flow cytometery analysis, which was performed using the BD Accuri C6 flow cytometer.

For cellular apoptosis assay hMSCs were cultures in 6 well plate, prior to treatment with MoS_2 and photothermal treatment as described above. After exposure to MoS_2 and photothermal for 24 hrs the cells were harvested via trypsinization and apoptosis assay was performed using the eBioscience Annexin V-FITC Apoptosis Detection Kit following the manufacture protocol and using a BD Accuri C6 flow cytometer.

Scratch assay using a pipette tip was used to evaluate wound healing-like migration. This was performed on a monolayer of hMSCs, human smooth muscle cells (hSMCs, Lonza CC-2579) and Michigan Cancer Foundation-7 (ATCC® HTB-22™) exposed to MoS_2 concentration and/or NIR exposure power for 3 hours as previously indicated. Using ImageJ (NIH) cell migration was quantified.

Integrin beta1 (CD29) expression was evaluated using CD29 Alexa Fluor® 647-conjugated antibody (R&D Biosystems) in combination with flow cytometry and fluorescence imaging. hMSCs were cultured in 6 well plates (for flow cytometry) and 24 well plates (for imaging) till they achieved 60% confluency prior to treatment with nanosheets(25 ug/mL and 100 ug/mL respectively) for a period of 24 hrs. Prior to fluorescence imaging cells were fixed, permeabilized, blocked and stained with anti CD29 antibody, Alexa Fluor® 488 phalloidin and DAPI, respectively.

RNA-seq. hMSCs were cultured and treated with low-intensity NIR exposure and/or exfoliated MoS_2 (25 μg/mL, 48 h). At the end of 7 days of culture, total RNA was extracted (High Pure RNA Isolation, Roche Life Sciences), and then converted into cDNA (>1 μg). Samples were run on an Illumina NovaSeq (NovaSeq 6000) utilizing TruSeqRNA preparation and 75 paired-end read length.

RNA-seq analysis. Sequenced reads were aligned to the human reference genome (hg38) using a RNA-seq aligner, Spliced Transcripts Alignment to a Reference (STAR)(5). Expression of a gene was determined by counting the number of uniquely mapped reads overlapping the coding exons normalized by gene length in fragments per kilobase per million (FPKM). Genes expressed with > 1 FPKM in at-least half of the samples of any condition were only used for the analysis. The gene expression read counts were modeled as a negative binomial distribution in generalized linear models (GLMs)(6) to determine the differentially expressed genes. All the analysis was performed using R. The GO enrichment analysis was done using GOStats Bioconductor package.(7) For network formation (Cytoscape)(8) and GO term enrichment (GeneMANIA, ClueGO), only genes with FDR-adjusted $P < 0.05$ were selected. REVIGO(9) was performed to visualize GO clustering.

3D Invasion Assay: 3D invasion experiments were established using 2.5 mg mL^{-1} type I collagen matrices containing 1×10^{-6} M S1P (Sigma, St Louis, MO), 40 ng mL^{-1} VEGF and bFGF. 3D gels were fabricated by thermally crosslinking the collagen solution at 37o C for 1hrsin a 96 well plate (100 ul of the solution was added per well). Following this endothelial cell were seeded at 30 000 cells per well in EGM2 supplemented media. After 2 hrs the cells were treated MoS_2 nanosheets (25μg/mL) and NIR stimulation as mentioned earlier. After 24 h incubation at 37 °C with 5% CO2, invading cells were fixed with 3% glutaraldehyde in phosphate buffered saline and stained with 0.1% toluidine blue/30% methanol. Results from at least three independent experiments are shown. For invasion distance, gels are sliced and laid on their side. Four random photographs were taken at 10X and 20X magnification. The distance migrated from the monolayer was recorded using side view images taken with a Zeiss AxioVert.A1 microscope. Images from each treatment group were included for the analysis. A structure is the furthest tip of a migrating cell. Peripheral sprouts were not included. ImageJ (NIH) software was then used to quantify invasion distance.

Western Blot Analysis. For Western blot analysis, cells were cultured under similar conditions as RNA-seq. Protein samples were isolated via a Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 100 mM Tris HCl, and 0.2% bromophenol blue). Gel electrophoresis (Mini Gel Tank; Invitrogen) was performed on protein samples, and subsequent gels were transferred (iBlot 2; Invitrogen) to a nitrocellulose membrane according to the manufacturer's protocol. The membranes were blocked with Superblock™ T20 blocking buffer for 30 min, and then processed using the iBind system (iBind; Invitrogen). ITGA7, FAK, ACTA2, Vinculin and Paxillin primary antibodies and HRP-conjugated secondary antibodies were purchased from either ABclonal or Proteintech, and incubation was performed per the manufacturer's protocols. Blots were developed (SuperSignal West Pico PLUS Chemiluminescent Substrate; Thermo Fisher)

and imaged via LI-COR 3600 CDigit Blot Scanner. Protein bands were quantified with LI-COR software.

Statistical Analysis. Determination of statistical significance between multiple groups was completed via analysis of variance (ANOVA) with Tukey method. Significant p-values were considered <0.05 unless otherwise noted. All analysis was completed in GraphPad Prism8 (San Diego, CA).

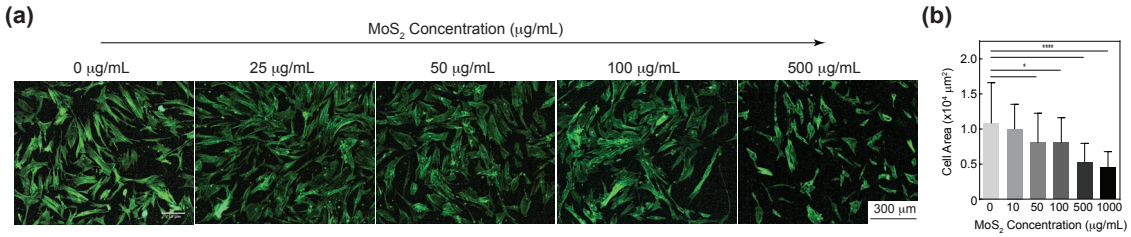

Figure S1: Cell spreading determined by quantifing individual cell area by treating hMSCs with different concentration of MoS_2.

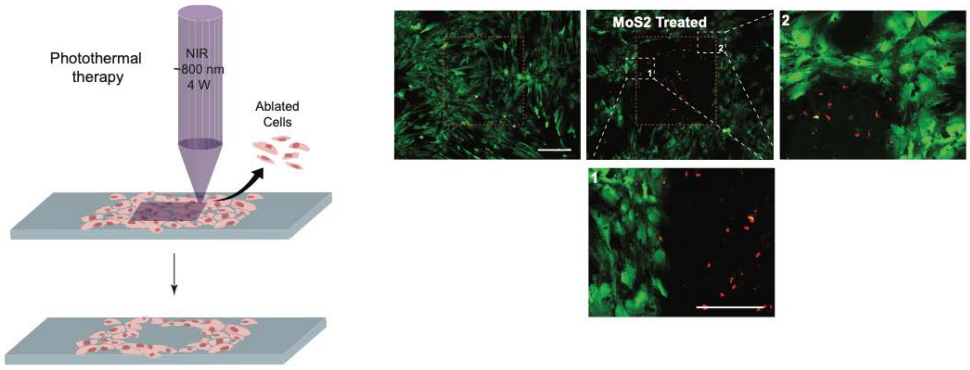

Figure S2: Photothermal effect of MoS_2 using high internsity NIR. Selected area can be abalated by targetting NIR to specifica area as demonstrated in the image.

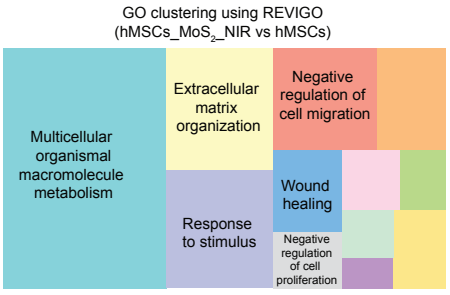

Figure S3: REVIGO analysis, a data management technique to reduce redundancies amongst terms from clustered genes, refined enrichments within the MoS_2/NIR treatment

group to macromolecule metabolism, ECM organization, response to stimulus, wound healing, and regulation of motility and proliferation.

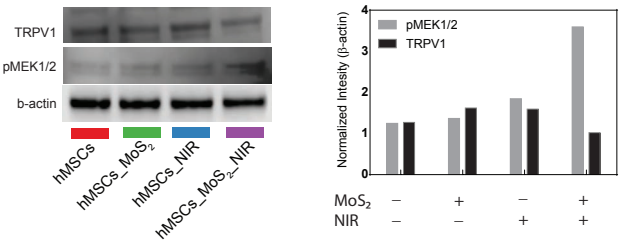

Figure S4: Effect of different treatments (NIR, MoS_2, and MoS_2_NIR) on Phospho-MEK1/2 and transient receptor potential cation channel subfamily V member 1 (TRPV1). MEK1 and MEK2, also called MAPK or Erk kinases, are dual-specificity protein kinases that function in a mitogen activated protein kinase (MAPK) cascade controlling cell growth and differentiation. Dual treatment (MoS_2 and NIR) result in significant increase in production of pMEK1/2, while no effect on TRPV1 was observed.

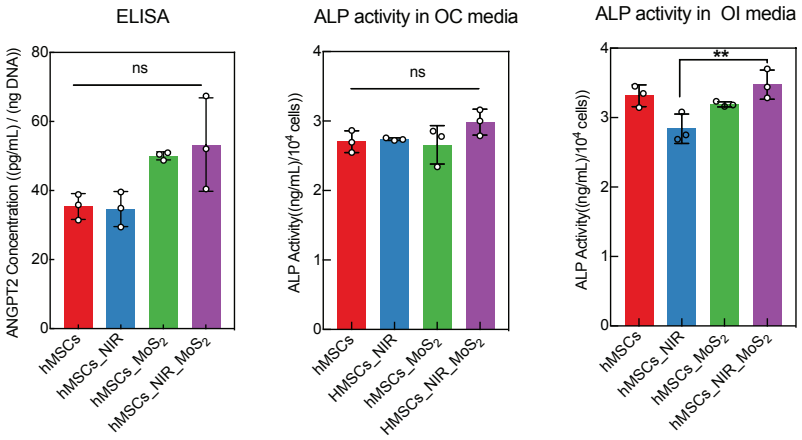

Figure S5: Effect of different treatments (NIR, MoS_2, and MoS_2_NIR) on pro-angiogenic factor - Angiopoietin 2 (ANGPT2) in normal growth media, as well as osteogenic differentiation marker - Alkaline Phosphatase (ALP) in osteoconductive (OC) and osteoinductive (OI) media. OC contain normal growth media along with beta glycerol phosphate and ascorbic acid, while OI contain normal growth media, beta glycerol phosphate, ascorbic acid and dexamethasone.

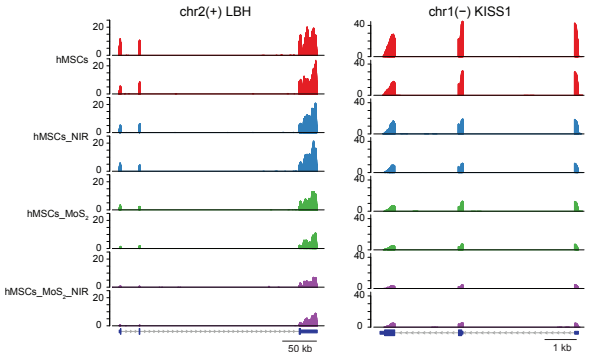

Figure S6: Gene track showing normalized mRNA expression of Limb Bud And Heart Development (*LBH*) and Kisspeptin-1 (*KISS1*) for hMSCs, hMSCs_NIR, hMSCs_ MoS_2, and hMSCs MoS_2_NIR.

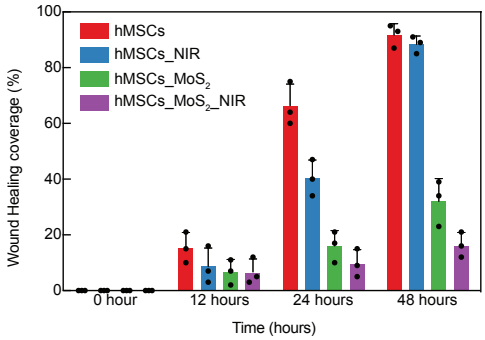

Figure S7 Scratch assay used to determine the effect of NIR, MoS_2 and MoS_2_NIR on migration, cell proliferation and wound healing of hMSCs. The cell migration across the wound area was determined after 12, 24 and 48 hours.

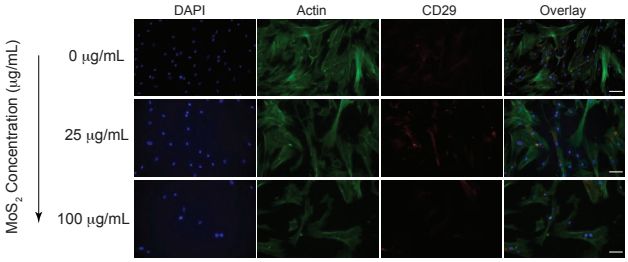

Figure S8: Confocal images to demonstrate staining for integrin beta 1 (CD29) after treatment with different concentration of MoS_2

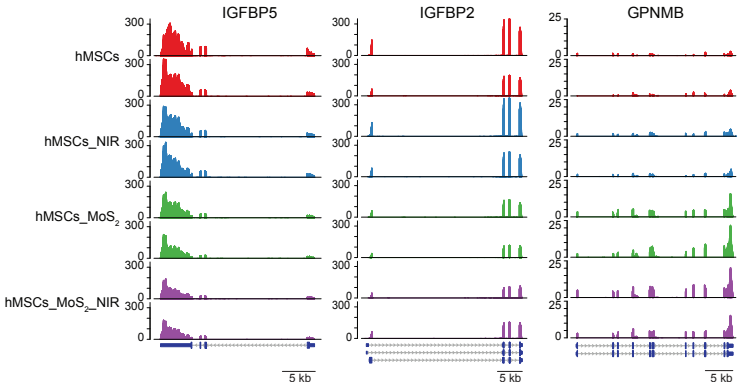

Figure S9: Gene track showing normalized mRNA expression of Insulin Like Growth Factor Binding Protein 5 (*IGFBP5*), Insulin Like Growth Factor Binding Protein 2 (*IGFBP2*) and Glycoprotein Nonmetastatic Melanoma Protein B (*GPNMB*) for hMSCs, hMSCs_NIR, hMSCs_ MoS_2, and hMSCs MoS_2_NIR.

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