# SUPPLEMENTAL MATERIAL

# Not just a marker: CD34 on human hematopoietic stem/progenitor cells dominates vascular selectin binding along with CD44

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**supplemental Materials and methods**

**Cells, enzymes, and reagents:**

*Cells:* Primary human CD34$^{pos}$ HSPCs harvested from umbilical cord blood (CD34$^{pos}$-UCB), healthy bone marrow (CD34$^{pos}$-BM), bone marrow from patients with newly diagnosed or relapsed acute myeloid leukemia (AML BM-CD34$^{pos}$), and mononuclear fractions of whole bone marrow were purchased from ALL CELLS (www.allcells.com). AML patient samples with newly diagnosed/relapsed AML [patient 1 (newly diagnosed): AML with genetic abnormalities, t(6;11)(q27;q13), >80% myeloid blasts; patient 2 (newly diagnosed): AML, patient clinical report was incomplete at time of collection, >50% myeloid blasts; patient 3 (relapsed): AML with genetic abnormalities, t(7;13?)(q22;q14), 95% monoblast/myeloid blasts and; patient 4 (relapsed): AML with genetic abnormalities, t(9;11)(p22;q13)]. KG1a, a human AML-derived HSPC-like CD34$^{pos}$ cell line, purchased from American Tissue Culture Collection (ATCC), was cultured in RPMI 1640, 10 % fetal bovine serum (FBS, GIBCO) and 100 U/ml penicillin/streptomycin (Invitrogen). Transgenic Chinese hamster ovary (CHO) cell lines stably expressing full-length human E-selectin (CHO-E), full-length human P-selectin (CHO-P), or the plasmid alone (CHO-Mock) were established and maintained as previously described.[1]

Specific populations of human HSPCs were isolated from bone marrow by washing with PBS/0.5% BSA and filtering cells through a 100μm cell strainer (BD Falcon), prior to the lineage depletion using the lineage cell depletion cocktail (including biotinylated antibodies: CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a) of monoclonal antibodies (mAbs; Miltenyi Biotec). The CD7 antibody was

also added to the lineage depletion cocktail in order to exclude lymphoid progenitors that are often found in the CD34$^{neg}$ fraction (eBioscience).[2] The cells were then separated using the depletion protocol (dep025) on the autoMACS cell-sorting machine (Miltenyi Biotec). The lineage negative (Lin$^{neg}$) fraction was then stained with FITC conjugated anti-human CD38 (IB6; R&D systems) and APC conjugated anti-human CD34 (AC136; Miltenyi Biotec) at a ratio of 10 µl mAbs /1x10$^{7}$ cells. The cells were then analyzed and sorted in the KAUST Bioscience Core Lab using a BD Influx™ sorter (Becton Dickinson). **Figure 1** depicts the sorting gates used. The purity of the sorted samples was consistently over 90%.

*Enzymes:* O-sialoglycoprotein endopeptidase (OSGE) was purchased from Cedarlane Laboratories, *Vibrio cholerae* neuraminidase from Roche and Peptide:N-Glycosidase F (PNGase F) from New England Biolabs. *Aerobacter aerogenes* sulfatase (Arylsulfatase) and bromelain (pineapple strain) were purchased from Sigma-Aldrich. All reagents used in Surface Plasmon Resonance (SPR) were purchased from GE Healthcare.

*Antibodies:* The following clones of CD34 antibodies were used: QBend-10 against class 2 mAb (Ms $IgG_1$; Novus biologica), 4H11 (Ms $IgG_1$; Exbio Praha), 581 (Ms $IgG_1$; BioLegend), 8G12 (Ms $IgG_1$; BD Pharmingen) and 563 (Ms $IgG_1$; BD Pharmingen) against class 3 and the C-terminus (C-18, Santa Cruz Biotechnology INC; EP373Y Novus biologica). Mouse IgG isotype control was purchased from Abcam. Recombinant mouse E- and P-selectin/human Ig chimera (E-Ig, P-Ig), mouse anti-human CD44 (2C5, Ms $IgG_{2a}$) and mouse anti-human CD43 (290111; Goat IgG) were purchased from R&D Systems. The following antibodies were purchased from BD Pharmingen (San Jose, CA): function blocking mouse anti-human E-selectin (HAE-1F; polyclonal IgG), rat anti-

human sLe$^x$ (HECA-452; Rat IgM), mouse anti-human PSGL-1 (KPL-1; Ms $IgG_1$), mouse anti-human CD44 (515; Ms $IgG_1$), mouse anti-human CD44 (Hermes-3; Ms IgG2a), mouse anti-human CD43 (1G10; Ms $IgG_1$), anti-mouse Cy-5, streptavidin Alexa Fluor 488 mouse $IgG_1$ isotype, mouse $IgG_{2a}$ isotype, mouse IgM isotype, rat IgG isotype, rat IgM isotype, PE streptavidin, biotinylated anti-rat IgM, goat anti-mouse Ig-HRP, and human $IgG_1$ isotype. Purified anti human-Galectin-9 (9M1-3; Ms $IgG_1$) was purchased from BioLegend and anti human Galectin-3 (A3A12; Ms $IgG_1$) was purchased from Abcam. The following secondary antibodies were purchased from Southern Biotech: rabbit anti-human IgG-biotin, goat anti-mouse IgG-HRP, goat anti-mouse IgM-PE, goat anti-rat IgG-PE, goat anti-mouse Ig-biotin, goat anti-rat Ig-HRP, and goat anti-human Ig-HRP. The Vybrant ® Lipid Raft Labeling kit was purchased from Thermo-Fisher scientific.

**Flow Cytometry:**

Aliquots of cells ($2 \times 10^5$ cells) were washed with PBS/2% FBS and incubated with primary mAbs or with isotype control mAbs (either unconjugated or fluorochrome conjugated). For E-Ig (10 µg/ml) staining, chimera buffer (HBSS/5mM HEPES/2mM $CaCl_2$/5% FBS) was used for all incubations, dilutions and washings. Then cells were incubated with isotype matched secondary fluorochrome-conjugated antibody and analyzed using FACS Canto (Beckman Dickenson). As a control for E-Ig staining, 20 mM EDTA was added to the chimera buffer.

**Enzyme treatment:**

For SPR analysis, either immunoprecipitates or whole cell lysates, that were prepared as described below at a density of $1x10^7$ cell/100 µl, were treated with the following enzymes: PNGase F (8 U/ml for 4 h) diluted with 50 mM phosphate buffer and 1% NP-40, OSGE (240 µg/ml for 4h) or neuraminidase (0.2 U/ml for 3 h). To prevent overall sulfation, $5x10^5$ cells/ml were incubated in a regular medium containing 150 mM sodium chlorate for 72 h following the treatment with bromelain (as described in the main Methods section) done to eliminate sulfated proteins on the cell surface. For tyrosine desulfation, KG1a whole cells lysates were treated or not (control) with arylsulfatase (5 U/ml) for 3 h. Equal amounts of CD34 were immunprecipitated from both treated and untreated samples and stained with either P-Ig or CD34 (EP373Y-mAb). Successful treatment was confirmed using PSGL-1 mAb on the whole cell lysate after treatment since the KPL-1 recognizes only sulfated epitopes.[3] For each enzyme digestion, a control was established by treating the lysates and immunoprecipitates with the same buffer and treatment conditions in the absence of the enzymes. All treatments were done at 37 $^{o}$C.

**Immunofluorescence Analysis:**

KG1a cells were cultured overnight on poly-L-ornithine-coated coverslips (Invitrogen). Attached cells were treated or not with E-Ig chimera (10 µg/ml) for 1h at 37$^{o}$C then washed with PBS containing 5% goat serum buffer, stained with AF594 conjugated Choleratoxin-β subunit (CTB, 10 µg/ml) for 30 min, crosslinked with anti-CTB for 30 min on ice, then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature (RT). After fixation, cells were blocked with blocking buffer (PBS containing 10% normal goat serum, 2% BSA), stained with anti-CD34 mAb (QBend-10, 5 µg/ml) in blocking buffer for 2 h at room temperature then incubated with isotype matched

secondary Abs (streptavidin (blue) toward biotinylated anti-human-Ig to detect E-Ig and Cy-5 anti-mouse IgG (red), 20 μg/ml, 30 min at 4 °C). Images of mounted cells (Invitrogen) were captured using Zeiss LSM 710 Axio confocal microscope (100× oil immersion lens) and analyzed for co-localization using ImarisV7 software (coloc tool). Cell surface labeling with secondary antibodies or isotype controls were used to determine the specificity of binding/background signal for specific antibodies (eg. HECA-452 and CD34) and human immunoglobulin (recombinant hIg1; Abcam) or secondary alone antibodies (anti-human Ig) were used to determine the specificity of binding for selectins (E-Ig or P-Ig).

**Hydrodynamic Flow Analysis of Selectin and Ligands Binding using Surface Plasmon Resonance (SPR):**

SPR binding experiments were performed using a Biacore T-100 system (GE Healthcare) at 25 °C. The system and flow cells were washed with the corresponding running buffer (filtered with 0.2 μm filter and degased) by conducting two priming steps. Immobilization of mAbs on a carboxy-methylated-5 (CM5) dextran sensor chip was performed using an amine coupling kit (BIAcore). According to manufacturer instruction, CM5 chip was activated with a 7 min injection of a 1:1 ratio mixture of N-hydroxysuccinimide (NHS) (0.1 M) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (0.4 M) at a flow rate of 5 μl/min. This was followed by mAb injection at a concentration of 20 μg/ml in 10 mM sodium acetate buffer (pH 5.0) at a flow rate of 10 μl/min to reach an immobilization levels between 4000-8000 Response Units (RU). The surface was then deactivated using 7 min injection of 1 M

ethanolamine hydrochloride at a flow rate of 5 µl/min. The immobilization steps were performed in HBS-EP buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 0.005% surfactant P20). The final exact RU for each immobilized mAb is specified in the text and/or corresponding figure legends. A control flow cell that was immobilized with an equal amount of the mAb-corresponding isotype-control was used to correct for the buffer's- bulk refractive index and nonspecific interactions with the surface and with the mAb. Further binding experiments were performed at a standard flow rate of 20 µl/min, unless otherwise specified, in the running buffer for E-Ig binding (1% Triton X-100, 50 mM NaCl, 50 mM Tris-base (pH 8.0) and 1 mM $CaCl_2$) and for P-Ig binding (0.05% P20, 150 mM NaCl, 2 mM $CaCl_2$, 50 mM Tris-base pH 8.0) for P-Ig binding. Whole cell lysates were prepared as described below but in the presence of higher NaCl concentration (250 mM). The lysates were injected over the immobilized Abs for a standard time of 700 s, unless otherwise specified. The flow cells were washed with running buffer for a standard time of 3 min, unless otherwise specified, to remove nonspecifically mAb-bound proteins.

In order to determine the specificity of the captured CD34, we recovered captured proteins from flow cells immobilized with 4H11-mAb. As a control, we recovered any captured protein from flow cells immobilized with $MsIgG_1$ isotype. The experiment was conducted in accordance with the recovery method in the BIacore T-100 software. Briefly, KG1a lysate was injected at 20 µl/min for 600 s followed by a buffer washing step for either 1- or 5-minutes at the same flow rate used in the binding experiment (20 µl/min). The captured proteins were incubated for 30 min with buffer containing (1%

Triton X-100, 50 mM NaCl, 50 mM Tris-base (pH 8.0) and 5 mM EDTA) and subsequently eluted in a total volume of 10 µl. The cycle of CD34 binding and elution was repeated five times and the total sample was analyzed by western-blot.

E-Ig was injected at 177 nM (diluted in the corresponding running buffer) for a standard time of 130 s, unless otherwise specified. The standard regeneration step for removing the bound E-Ig was a 130 s injection of running buffer containing 1 M NaCl at a flow rate of 45 µl/min. The glycoprotease experiments were performed beginning with the enzyme-treated lysates since only minimal (to no) binding of CD34 to E-Ig was observed. Then in addition to the standard regeneration step, an extra step to remove the bound CD34 from corresponding mAb by washing with running buffer for 1 h.

Data analysis was performed using Biacore evaluation software. To make a comparative analysis among different lysate injections, the entire sensogram was multiplied by a normalization factor that was defined based on its relative RU just prior to E-selectin injection to the RU in the flow cell with the highest accumulated CD34. The relationship between the amount of immobilized proteins ($RU_{ligand}$) and the bound protein ($RU_{analyte}$) and their stoichiometry were calculated based on equation (1):

$$\mathrm{RU}_{ligand} = \mathrm{RU}_{analyte} \text{ x } \left( \frac{\text{molecular weight of ligand}}{\text{molecular weight of analyte}} \text{ x valency}_{ligand} \right).$$

Note that the calculated ligand and analyte molecular weights are based on apparent molecular weight determined by SDS-PAGE gel electrophoresis (120 kDa for CD34, 80kDa for CD44, 130 kDa for CD43 and 240 kDa for dimeric PSGL-1).

To determine the dissociation binding constant ($K_D$), consecutive injections of E-Ig at 15 µl/min for 240 s each at concentrations indicated in the figures was performed over

captured E-selLs (CD34, CD44, CD43, or PSGL-1). Injections for each E-Ig concentration were followed by a 60 s buffer washing step without a regeneration step in-between. The maximum RU reached prior to the start of the wash with buffer ($RU_{max}$), where a steady-state condition was nearly met, were plotted against the E-Ig concentration and fitted using the steady-state model provided by the Biacore evaluation software to determine the $K_D$ value. The dissociation rate constant ($k_{off}$) of the mAb·captured-ligand complex or the apparent dissociation rate constant ($k_{off\text{-}apparent}$) of the interaction of mAb·captured-ligand with E-Ig from a single concentration-type binding study was calculated by fitting the stable phase in the buffer washing step using the Biacore evaluation software. $k_{\text{on-apparent}}$ was calculated based on the determined $K_D$ and the $k_{\text{off-apparent}}$ values.

**Immunoprecipitation (IP) and Western blot Analysis:**

Cells (human $CD34^+$ BM HSPCs and KG1a cells) were lysed using (1% Triton X-100 in 150 mM NaCl, 50 mM Tris-HCI; pH 7.4, 5 mM EDTA, 20 μg/ml PMSF and protease inhibitor cocktail tablet (Roche, IN)). For E-selL (eg. CD44, CD34, etc) IPs, lysates were pre-cleared with Protein G agarose beads (Life Technologies) 3 times (20 min each) followed by incubation with the corresponding antibody. For CD34 IPs a mixture of mAbs (4H11; QBend-10; EP373Y) was used unless otherwise described in the legend. A mixture of 2C5 and 515 were used for CD44 IPs. CD43 mAb, Clone # 290111, was used to IP CD43 while the KPL-1 clone was used to IP PSGL-1. For IPs using E-Ig, lysates were made using lysis buffer containing 2 mM $CaCl_2$ but lacking EDTA for 1 h at 4 $^oC$. The lysate was spun down at high speed in a refrigerated micro-centrifuge (Eppendorf), precleared with Protein G Agarose beads (Life Technologies), and then incubated with

antibodies or recombinant protein (E-Ig, P-Ig) (each at 3 μg, 2 h at 4 °C). Then this mixture was incubated with 30 μl beads for 3 h at 4°C under constant rotation, washed with lysis buffer containing 2% BSA followed by lysis buffer only, diluted in reducing sample buffer, boiled, subjected to 4-20% Mini-Protean TGX Precast gels (Bio-Rad Laboratories, Hercules, CA), transferred to PVDF membrane, and immunostained with the specified antibodies or recombinant protein (E-Ig/ P-Ig) as described previously.[1] Additional control blots were stained with secondary antibody only for both immunoprecipitated CD34 and CD44 blots as well as by using isotype controls. Control blots for E-Ig staining were stained with 20 mM EDTA to confirm binding specificity as well as with h-IgG1 recombinant protein followed by the secondary antibody. Exhaustive E-Ig IP was done from each round as described above. Between each antibody treatment, two rounds of beads were used to remove any potential residual antibody from the previous IP treatment; western-blot of these samples yielded no signal with any antibody sequences tested, suggesting that the prior IP was complete with no residual antibody. In order to have a quantitative measurement of Western blot bands, NIH ImageJ was used where the area of each band was analyzed using the gel analyzer tool. Then the band density was normalized to selected control as mentioned in the figure legend.

**Stamper-Woodruff assay:**

CD34 immunoprecipitates from human BM-CD34$^{pos}$ cells ($3.3 \times 10^5$ cell) were diluted in the sample-reducing buffer, boiled, spotted onto glass slides and allowed to dry at room temperature. Spots were then fixed with 3% glutaraldehyde, blocked with 0.2M L-lysine, and then incubated on slides in RPMI 1640, 5 mM $CaCl_2$ and 2% FBS until the analysis.

CHO-E or CHO-P cells were overlaid onto these fixed immunoprecipitates and rotated with an orbital shaker at 80 rpm for 30 min at 4°C. The number of adherent cells was quantified by light microscopy using a 20× ocular lens from a minimum of eight fields/slide from two slides. Nonspecific adhesion was assessed by flushing CHO-E or CHO-P cells suspended in the same buffer but supplemented with 5 mM EDTA.

**Blot-rolling assay:**

For this assay, we followed a technique previously described.[1,4,5] Briefly, Western blots of CD34 (QBend-10, 4H11 and EP373Y), CD44 (2C5 and 515), CD43 (290111), and PSGL-1 (KPL-1) immunoprecipitates were stained with anti-CLA (HECA-452) and rendered translucent by immersion in Dulbecco's modified Eagle's medium (DMEM) with 10% glycerol. CHO-E cells were resuspended ($5x10^6$ cells/mL) in DMEM containing 2 mM $CaCl_2$ and 10% glycerol. The blots were placed within a parallel plate flow chamber, and CHO-E, CHO-P, and CHO-Mock cells were perfused at a shear stress of 0.25 dyne/$cm^2$ with an adjustment in the volumetric flow rate to account for the increase in viscosity due to the presence of 10% glycerol in the flow medium. The average number of interacting cells/field was quantified using a 20x ocular lens from a minimum of four fields/membrane of seven independent experiments. Nonspecific adhesions were assessed by flushing either CHO-E or CHO-P cells suspended in the same buffer but with an added 5mM EDTA or using CHO-Mock cells. Binding above these nonspecific interactions was reported. Rolling cells were tracked using Imaris V7 software (Bitplane) as described previously in.[1]

**Transmission Electron Microscopy (TEM) Studies:**

Kg1a Cells (150 million cell) were either transfected with scrambled negative control siRNA or with CD34 siRNA (CD34-KD). 48 h post transfection cells were fixed with 2.5% Glutaraldehyde. Centrifuged cell pellets were then reconstituted in a buffer containing Sodium cacodylate (0.1 M)/ Glutaraldehyde (2.5%). Samples were then stained with 2% Osmium followed by dehydration using an increasing concentration of ethanol. Resin infiltration was done using an Epon/acetone mixture of gradually increasing concentration. Resin molds of the samples were sliced with an ultramicrotome Leica UC6 and the slices were placed on grids and were stained with uranyl acetate followed by lead citrate. TEM images of the grids were acquired using Tecnai Spirit TWIN (FEI), at 120 kV, equipped with an ORIUS SC1000 camera (Gatan).

**supplemental Figures**

**supplemental Figure 1.** CD44 immunoprecipitates were prepared from lysates of $CD34^{pos}$ bone marrow cells ($CD34^{pos}$-BM), $Lin^{neg}CD38^{neg}CD34^{pos}$ and $Lin^{neg}CD38^{neg}CD34^{neg}$ populations isolated from human bone marrow and subjected to Western blot analysis. Membranes were blotted with CD44 (*top*), E-Ig (*middle*), or HECA-452 (*bottom*) followed by isotype-matched HRP-conjugated mAb for visualization. This is representative of $n=3$ independent experiments.

**supplemental Figure 2**. Neither Galectin-3 nor Galectin-9 were co-immunoprecipitated with CD34 from $CD34^{pos}$ cells. **(A)** KG1a whole cell lysates were prepared for Western blot analysis and blots were stained for Galectin-3 and Galectin-9 in order to determine their expected molecular weights (red arrows) in our cells of interest. **(B)** KG1a and $CD34^{pos}$-BM cells (5 million) were lysed and normalized for protein concentration. CD34 was immunoprecipitated from lysates using anti-human CD34 antibodies (QBend-10 and 563) and subjected to Western blot analysis. Blots were then stained for CD34 (*left*), Galectin-9 (*middle*) or Galectin-3 (*right*). CD34 was detected while neither Galectin-9 nor Galectin-3 was detected at the indicated molecular weights. The bands observed in the CD34, Gelectin-3 and Galectin-9 blots represent the chains of the antibodies used to immunoprecipitate CD34 (black arrows; ~50kDa, ~75kDa and ~100kDa as determined by a performing a $2^{o}$ antibody control blot using goat anti-rabbit IgG-HRP). **(C)** To confirm the functionality of the Galectin-9 and Galectin-3 antibodies, lysates of Jurkat clone E6-1 cells and HeLa cells were subjected to Western blot analysis and blots were stained for either Galectin-9 (Jurkat clone E6-1) or Galectin-3 (HeLa). The results are representative of $n=3$ independent experiments.

**supplemental Figure 3**. Addition of recombinant E-selectin to cultures of KG1a cells leads to increased cellular aggregation. KG1a cells were incubated with E-Ig, h-Ig (control) or untreated (not shown) for 1h at 37$^{o}$C then imaged prior to washing and preparing the cells for the immunofluorescence studies outlined in **Figure 2**. Note that since there was no difference between the h-Ig treated and untreated controls, the imaging was done with untreated controls following the first experiments.

## References


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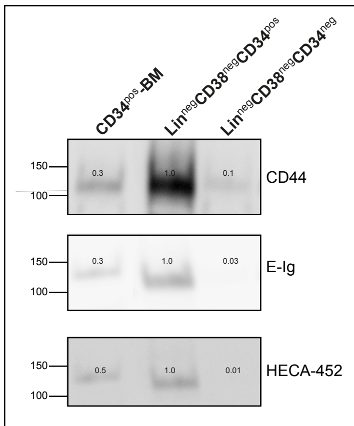
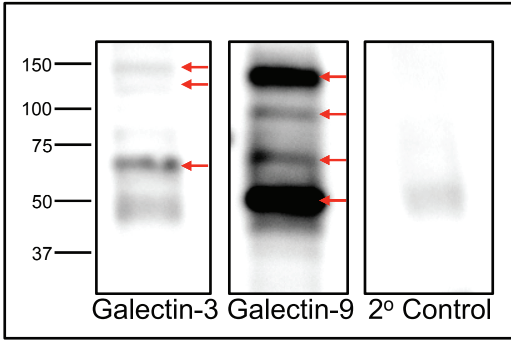
5. Sackstein R, Fuhlbrigge R. Western blot analysis of adhesive interactions under fluid shear conditions: the blot rolling assay. *Methods Mol Biol*. 2009;536:343-354.

## supplemental Figure 1:



**supplemental Figure 2:**

(A)

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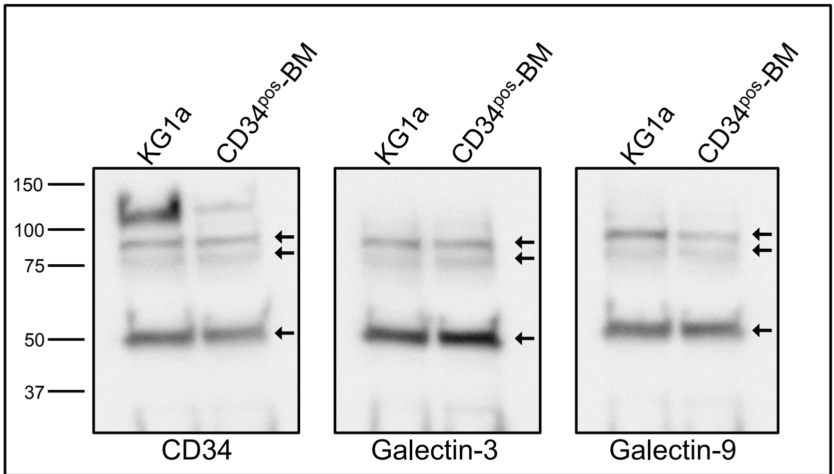


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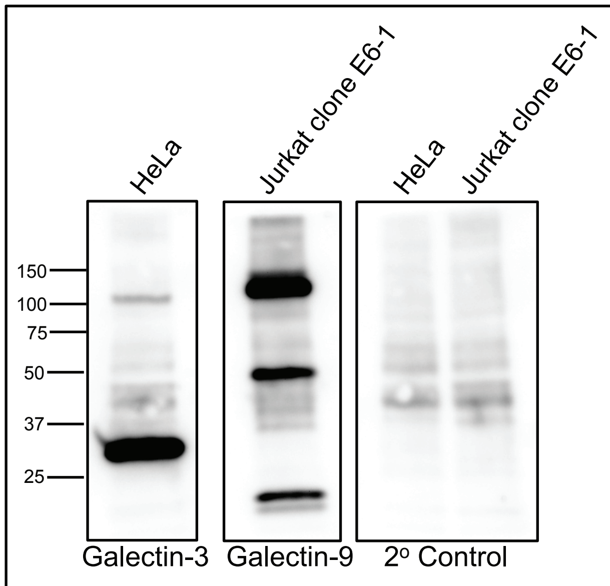

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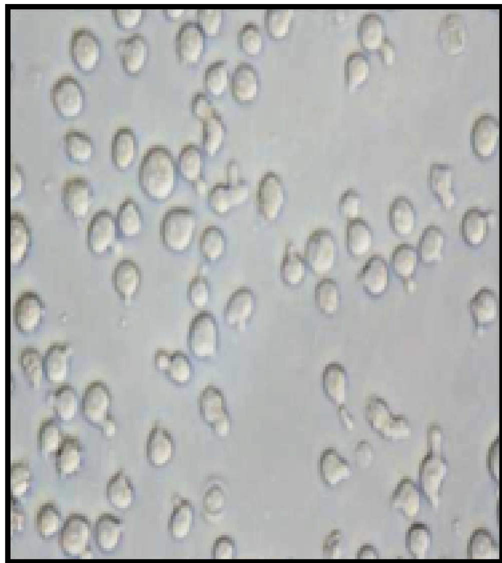
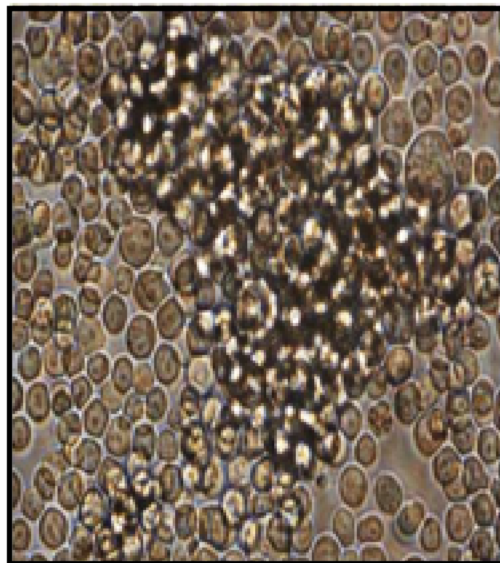


**Control**



**E-Ig treated**