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Investigating the role of CD44 and hyaluronate in embryo-epithelial interaction using an in-vitro model

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Running title: CD44 – hyaluronate interaction at implantation

Abstract

Implantation failure is an important impediment to increasing success rates in assisted reproductive technologies (ART). Knowledge of the cascade of morphological and molecular events at implantation remains limited. Cell surface CD44 and hyaluronate (HA) have been reported in the uterus, but a role in intercellular interaction at implantation remains to be evaluated. Mouse embryos were co-cultured with human Ishikawa endometrial epithelial monolayers over two days. Attachment was tenuous during the first 24 hrs, after which it became stable, leading to breaching of the monolayer. The effects of enzymatically reducing the density of HA, or introducing a function-blocking antibody to CD44, were monitored during progression from weak to stable embryonic attachment. Hyaluronidase-mediated removal of surface HA from the epithelial cells enhanced the speed of attachment, while a similar treatment of embryos had no effect. The antibody to CD44 caused retardation of initial attachment. These results suggest that CD44-HA binding could be employed by embryos during initial docking, but the persistence of HA in epithelial cells might be detrimental to later stages of implantation by retarding attainment of stable attachment.

Keywords: CD44, hyaluronate, implantation, endometrium, embryo adhesion.

Introduction

CD44 is a cell surface glycoprotein that acts as a receptor for hyaluronan (HA) as well as other ligands including osteopontin (OPN), collagens and matrix metalloproteinase 9 (Misra *et al.*, 2015, Senbanjo and Chellaiah, 2017). CD44 is present in a wide range of cells, with a complex pattern of splice variants and glycoforms, including the trophectoderm of both human and mouse blastocysts (Campbell *et al.*, 1995, Lu *et al.*, 2002) and endometrial tissue. In the mid secretory phase, when embryo implantation

occurs, CD44 is found at the lateral and apical surface of both glandular and luminal epithelial cells (Afify *et al.*, 2006, Albers *et al.*, 1995, Behzad *et al.*, 1994, Fujita *et al.*, 1994, Griffith *et al.*, 2010, Saegusa *et al.*, 1998, Saegusa and Okayasu, 1998). Ligand binding to CD44 leads to changes in cell motility, gene expression and growth (Senbanjo and Chellaiah, 2017). The fucosyl transferase FUT4 catalyses the addition of terminal α1.3-fucosyl residues to glycan on CD44, leading in turn to activation of the Wnt/β-catenin signalling pathway (Zheng *et al.*, 2017), which is associated with endometrial receptivity to implantation (Mohamed *et al.*, 2005), though an upstream ligand sensitive to glycoform has not been identified.

HA is present in uterine fluid and on the surface of the endometrial epithelium (Fouladi-Nashta *et al.*, 2017). Treatment of mouse embryos with HA promoted implantation (Gardner *et al*., 1999), and HA-containing embryo transfer medium used in ART has been reported to improve implantation and increase live birth rates in humans (Bontekoe *et al.*, 2014). CD44-HA interactions have therefore been implicated in embryo attachment during the early stages of implantation. HA is proposed to bridge between embryo and endometrial epithelium through CD44 (and possibly other receptors), while OPN dimers (Goldsmith *et al*., 2002) may bridge CD44 and/or integrin αvβ3. OPN is a strong candidate adhesion molecule for implantation (Johnson *et al*., 2014) and we have previously shown that integrin αvβ3-OPN interactions contribute to embryo attachment to epithelium in vitro (Kang *et al*., 2014). Recent experimental manipulation of HA in the sheep uterus however suggests that endometrial HA may act to inhibit implantation (Fouladi-Nashta *et al*., 2017, Marei *et al*., 2017).

We have used Ishikawa cells as a model endometrial epithelium for examining interaction with blastocyst stage embryos (Ruane *et al*., 2017, Ruane *et al*., 2018,

Singh *et al*., 2010). When embryos are transferred to confluent Ishikawa cell monolayers, initial attachment to the apical surface is followed by breaching and trophoblast outgrowth. A proteomic profile of glycoproteins exposed at the apical surface of confluent, polarised Ishikawa cells included CD44 (Aplin and Ruane, 2017, Singh and Aplin, 2015, Singh *et al*., 2010), verifying that these cells are suitable for evaluating its biological activity in this context. Here we examine the effects of blocking CD44 as well as stripping cell surface HA on attachment of mouse embryos.

Materials and Methods

Cell culture

Ishikawa cells (ECACC 99040201) were maintained at 37ºC, 95% air and 5% CO2 in DMEM (Sigma) containing 10% fetal bovine serum (Sigma), 2mM L-glutamine, 100µg/ml streptomycin and 100IU/ml penicillin (Sigma). Cells were grown on 2% Matrigel-coated 13mm glass coverslips (Sigma) in 24-well plates (Corning) up to passage 25.

Mouse embryos

Experiments were carried out under UK Home Office project license PPL 70/07838, and authorised by the Animal Welfare and Ethical Review Board of the University of Manchester, according to the Animal Act, 1986. Eight-week-old CD1 female mice (Charles River) were superovulated (by intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin (Intervet), followed by 5 IU human chorionic gonadotrophin (Intervet) 46 hrs later) and time-mated. The 2-cell embryos were flushed from the oviduct at E (embryo day) 1.5. Embryos were cultured in KSOM medium (Millipore) containing 0.4% BSA (Sigma) under oil (Vitrolife) to E4.5 then

artificially hatched from the zona pellucidae using acid Tyrode's solution (pH 2.5) (Sigma).

Cell spreading assay

Flat-bottom 48-well plates (Corning) were left uncoated or coated overnight with 5µg/ml osteopontin (R&D Systems), bovine serum albumin (BSA, Sigma) or poly-L-lysine (Sigma). Wells were then exposed to a solution of 1mg/ml heat-denatured BSA (Sigma) for 1 hr. Endometrial cells were trypsinised and incubated with various concentrations of antibody (H-300-L rabbit anti-CD44 (Santa Cruz) or IgG from rabbit serum as negative control, (Sigma); 5, 10 or 20µg/ml in serum-free medium (Teramoto *et al*., 2005) for 5 min at room temperature then plated into wells at 2000 cells/well. Cells were cultured for 1 hr then imaged using phase contrast microscopy and analysed using ImageJ.

In-vitro attachment assay

Confluent endometrial cells were incubated in serum-free medium (DMEM, 2mM L-glutamine, 100µg/ml streptomycin and 100IU/ml penicillin) for 24 hrs prior to co-culture with hatched E4.5 mouse blastocysts (3 per well), as previously described (Ruane *et al.*, 2017).

In some experiments, medium was spiked with antibody (H-300-L rabbit anti-CD44 or IgG from rabbit serum) at 20µg/ml (determined by data from the cell spreading inhibition assay), either just prior to addition of E4.5 embryos or 24 hrs later just prior to detachment of E5.5 embryos, by gently flushing with 60µl medium. Mouse embryos flushed at day 4.5 require 28 hrs of incubation with cells to activate them for stable attachment; flushing restored all embryos to an unattached state at the start of the

antibody incubation period. Other experiments involved pre-treatment of embryos and / or Ishikawa cells with hyaluronidase prior to co-culture. Here hatched E4.5 blastocysts were cultured in KSOM, 0.4% BSA to E5.5, then incubated with, or without, 500 IU/ml hyaluronidase (Sigma) in KSOM, 0.4% BSA for 30 minutes before transfer onto treated or untreated cells. Ishikawa cells were treated by culturing as usual for 24 hrs, removing and retaining the conditioned medium during a 30 min incubation with 500 IU/ml hyaluronidase (in fresh serum-free culture medium), then replacing the retained medium before adding treated or untreated E5.5 embryos.

In all experiments, attachment stability was assessed as previously described (Ruane *et al.*, 2017), every 4 hrs during the first 12 hrs of co-culture then at 24, 28, 32, 36 hrs and finally at 48 hrs (E6.5 of mouse embryo development) using an inverted phase contrast microscope (Evos XL Core). Co-cultures were then fixed with 4% PFA for 20 min at room temperature and stored under PBS at 4°C.

Single embryo fluorescence staining

Hatched E4.5 embryos were fixed in a staining solution (3% BSA in PBS) containing 1% PFA for 20 min, quenched in 50mM ammonium chloride for 5 min, then permeabilised using 0.5% Triton-X100 PBS solution for 6 min. Blastocysts were incubated overnight at 4°C in a 25µl drop of staining solution containing H-300-L rabbit anti-CD44 or IgG from rabbit serum under mineral oil (Sigma) followed by staining solution containing an Alexa Fluor 488-labelled secondary antibody (Life Technologies) and 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 1 hr. Embryos were mounted in a poly-L lysine-coated chamber of 3% 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma) in PBS.

Endometrial/endothelial cells and embryo attachment staining

PFA-fixed cells and co-cultures were quenched with 50mM ammonium chloride before permeabilisation in 0.5% Triton-X100 PBS. Mouse monoclonal anti-CD44 antibody (5F12, NeoMarkers Fremont) and IgG from mouse serum (negative control, Sigma) and Alexa Fluor 488-labelled secondary antibody (Life Technologies) were used to stain Ishikawa cells and attachment sites. Rabbit polyclonal (H-300-L, Santa Cruz) was used in some experiments. Hyaluronan was visualised by incubating samples for 3 hrs at room temperature with a biotinylated hyaluronan binding protein (Biotin-HABP, Amsbio), followed by streptavidin-fluorescein isothiocyanate for 1 hr at room temperature. Nuclear and actin stains were performed using a mixture of DAPI (Sigma) and Alexa Fluor® 568-coupled Phalloidin (Invitrogen). Coverslips with cell layers were mounted upside down on a microscope slide in a drop of Mowiol 4-88 mounting medium (Sigma) containing 3% DABCO. Coverslips with embryo-Ishikawa cells were mounted a chamber of 3% DABCO in PBS to maintain the 3D structure of the attachment sites.

Fluorescence microscopy

Fluorescence microscopy images were taken with an inverted Zeiss microscope, Zen 2.0 software and the Apotome 2 module, and analysed with ImageJ. Z-series optical sections of cells were obtained at the minimum of 0.24µm increments for 40X and 63X objectives. Z-stacks of embryo and embryo attachment sites were obtained at 1-2µm increments at 40X for a maximal distance of 60µm.

Statistics

Data generated from embryo attachment assays and cell spreading assays are represented as mean ± the standard error of the mean or median and interquartile range. Statistical analysis using Prism software (Graph-Pad, USA) included the 2-way

ANOVA test followed by Bonferroni's multiple comparisons post-hoc test or non-parametric Wilcoxon, Mann-Whitney & Friedman tests.

Results

CD44 immunoreactivity in blastocysts and Ishikawa cells

The rabbit polyclonal antibody H300 against CD44 showed heterogeneous binding in the trophectoderm of chemically hatched mouse blastocysts (Figure 1A-C). As previously reported (Behzad *et al.*, 1994, Singh *et al.*, 2010), Ishikawa cells express CD44. The monoclonal antibody 5F12, which did not show any reactivity with mouse embryos, revealed linear lateral distribution of immunoreactivity as well as more diffuse punctate staining in Ishikawa cells, with considerable intercellular variation (Figure 1D). Heterogeneous immunoreactivity was in ruffles at the apical surface (Figure 1E). Variation of culture conditions, including the presence and absence of serum and/or matrigel as a substrate, did not cause significant changes (not shown).

CD44 immunoreactivity in attachment sites in vitro

Transfer of hatched E4.5 mouse blastocysts to Ishikawa epithelial cell monolayers is followed by an initial period (28 hrs) of weak and reversible attachment that initiates the activation required for embryos to progress, over the next 20 hrs, from stable attachment to breaching and displacement of the underlying cells (Ruane *et al.*, 2017). Figure 2A shows 5F12 reactivity in cells surrounding an attachment site with no trophoblast invasion, whereas in Figure 2B, the trophoblast has breached the subjacent cell layer and is beginning to laterally invade, with CD44-positive epithelial cells crowded together in adjacent locations. Figure 2C surveys a subsequent stage in which trophoblast giant cells with prominent stress fibres are apparent spreading over

the substrate. Intensity scans of such sites showed no significant change in CD44 immunoreactivity in cells adjacent to the embryo either at this or earlier pre-breaching stages of attachment (not shown). Note this antibody does not detect staining in cells in adherent embryos.

Function-blocking antibody to CD44 can delay attachment

To test the hypothesis that CD44 might mediate attachment of blastocysts to epithelial cell layers, a function-blocking antibody, H300, was introduced into co-cultures. HA does not bind to culture plastic, so the potency of H300 as an inhibitor of CD44 function in Ishikawa cells was examined using an adhesion assay which monitors the capacity of cells to spread on dishes coated with OPN (Figure 3A,B). Inclusion of H300 resulted in partial inhibition (~ 55%) of spreading when cells were plated on OPN but the antibody had no effect when a control substrate containing poly-L-lysine was used.

Alterations to embryo behaviour in response to antibody inhibition of CD44 function were evaluated using a stability scale in which unattached and weakly attached embryos can be reliably distinguished from those achieving intermediate or fully stable attachment (Ruane *et al.*, 2017). Unattached embryos move across the monolayer when disturbed, while weakly and intermediately attached embryos are identifiable by high and low levels of oscillation, respectively, about an attachment point. Stably attached embryos do not oscillate. Combining intermediate and stable attachment scores produces a measure of irreversible attachment (Ruane *et al.*, 2017).

Introducing antibody to cultures just prior to the addition of E4.5 embryos impacted on weak attachment in the first 8 hrs, and inhibited the level of stable attachment levels at 32 hrs co-culture (Figure 3C,D). In a second series of experiments, the antibody was added after 24 hrs co-culture when embryos were at E5.5 and beginning to advance

from weak to stable attachment. Weakly adherent embryos were mechanically detached at the time of antibody addition, which we have previously demonstrated does not impact on their ability to progress to stable attachment (Ruane *et al*., 2017). Antibody spiking at this time point reduced total attachment levels between 28 and 32 hrs, though by 36 hrs the treated embryos had caught up with controls. Moreover, a trend towards reduced stable attachment was observed from 28-36 hrs with significant inhibition of stable attachment at 36 hrs (Figure 3E,F).

A role for endometrial hyaluronan in early embryo attachment

A fluorescent conjugate of the HA-binding domain of versican applied to fixed Ishikawa cells revealed prominent fluorescence, demonstrating HA localisation at the apical surface, where embryos initially dock (Figure 4A). After treatment with the enzyme Hyal2, which cleaves high molecular mass HA into smaller fragments, cells showed a negligible level of fluorescence (Figure 4B,C). There was no change in CD44 distribution in the treated cells (Figure 4D,E).

We then went on to investigate whether this apical surface-localised HA might contribute to the attachment reaction. We carried out co-cultures from E5.5, using cells and/or embryos pre-treated with Hyal2. Embryos stably attached more rapidly to cells that had been treated with the enzyme, the difference being apparent between 28 and 32 hrs; by 48 hrs the embryos had attached as stably as in untreated controls (Figure 4F,G). Comparing total attachment levels with those of stable attachment demonstrated that loss of HA has little effect on weak attachment at this embryonic stage (Figure 4F,G). Treating embryos with Hyal2 had no effect on attachment kinetics (Figure 4F,G).

Discussion

Our model system allows a detailed analysis of the kinetics of embryo attachment, which progresses from a reversible weak stage, with activation of trophoblast gene expression, to an irreversible stable stage that rapidly progresses to epithelial breaching (Kang *et al.,* 2014, Ruane *et al.*, 2017). CD44 immunoreactivity in Ishikawa cells and blastocysts is consistent with our previously reported analysis of the apical Ishikawa glycoproteome (Singh and Aplin, 2015) and matches CD44 localisation in human and murine blastocysts (Campbell *et al.*, 1995; Lu *et al.*, 2002). Data herein reveal that the presence of a function-blocking CD44 antibody leads to a delay in the progression of embryos to a stably attached state. Additionally, we found that enzymatic degradation of the CD44 ligand HA from the apical surface of Ishikawa cell layers, but not the blastocyst, hastened the attainment of stable attachment, with minimal effects on weak attachment. Together these data suggest that CD44 contributes to weak embryo attachment in a HA-independent manner while endometrial HA acts as a brake on progression to stable attachment.

Reflecting these data, pharmacological inhibition of uterine HA synthesis in sheep leads to its disappearance from the uterine apical epithelial surface and a corresponding increase in embryo attachment. Conversely, infusion of HA into the lumen inhibits implantation (Marei *et al.*, 2017). HA may therefore act analogously to the functions of mucins MUC1 and MUC16 (Aplin, 2000, Dharmaraj *et al.*, 2014, Gipson *et al.*, 2008, Hey *et al.*, 1994, Meseguer *et al.*, 2001). Moreover, embryonic activity causing loss of HA from the epithelial surface, as seen for MUC1 (Meseguer *et al.*, 2001, Singh *et al.*, 2010), may underlie the progression to stable attachment.

The use of HA as a supplement at the time of embryo transfer has received considerable attention in ART (Fouladi-Nashta *et al.*, 2017, Singh *et al.*, 2015), and

there is evidence to suggest that exogenous HA can bind to the luminal apical epithelial cell membrane (Marei *et al.*, 2017). Supplementation would be justified only if HA can be demonstrated to have a role in improving embryo viability, acquisition of blastocyst adhesion competence or supporting development that precedes the interaction with maternal epithelium at the start of implantation. Embryo development and viability were found to be improved after culture in HA-supplemented media in humans (Simon *et al*., 2003) and in other animal models (Gardner *et al*., 1999, Romek *et al*., 2017, Lane *et al*., 2003). Exogenous HA may actually delay rather than promote implantation in humans, and this impact on timing could underlie the beneficial effects of such transfer medium by allowing acclimatisation of the blastocyst to the uterine environment before implantation, or indeed synchronising a delayed window of receptivity with the implantation-ready blastocyst. HA-enriched transfer medium has also been shown to be beneficial during cleavage-stage embryo transfer (Urman *et al*., 2008; Nakagawa *et al*., 2012), implying that exogenous HA impacts upon embryo development or the acquisition of a receptive endometrium. However, the beneficial effect of HA-supplementation embryo transfer medium on implantation rate and clinical pregnancy is controversial (Simon *et al*., 2003, Fancsovits *et al*., 2015).

The presence of CD44 at the blastocyst-uterine interface is not essential for implantation in mice: CD44 null mice are fertile and viable with no morphological defect (Schmits *et al*., 1997, Protin *et al*., 1999). In these studies, CD44 null offspring from heterozygotic matings followed Mendelian rules, and their fertility status was reported, though not shown, based on breeding of the F_1 generation. Another HA-binding receptor, RHAMM, is expressed both in blastocysts (Choudhary *et al*., 2009) and endometrium (Rein *et al*., 2003, Ozbilgin *et al*., 2012), and could potentially compensate for the absence of CD44 (Nedvetzki *et al*., 2004, Naor *et al*., 2007, Toole,

2009), but it has not been investigated in CD44-null animals. Further investigations are necessary to determine the role of RHAMM in embryo attachment at implantation.

The anti-CD44 antibody we used has been shown to block binding of OPN to the N-terminus (Teramoto *et al*., 2005). Since this is also the major binding site in CD44 for HA, the antibody is likely to impair HA binding (Banerji *et al.*, 2007, Peach *et al.,* 1993). In the cell spreading assay, targeting CD44 was not expected to achieve a full inhibition as the endometrial cells express integrins also known to interact with OPN (Kang *et al.*, 2014). Furthermore, our HA clearance data suggest endogenous OPN is the more likely ligand involved in CD44-mediated early attachment between trophectoderm and endometrial epithelial cells (Kang *et al.*, 2014). This study provides evidence of a role for the CD44-OPN-HA axis in timely progression from weak (CD44-OPN) to stable (loss of HA) attachment, which we believe is important for the development of the invasive trophoblast required for the establishment of pregnancy (Ruane *et al*., 2017). If the observations were to translate to human embryos implanting in vivo, a delay of a few hours towards the end of the receptive phase might lead to failure to rescue the corpus luteum, and subsequent loss of the pregnancy (Baird *et al.*, 1991). Conversely, rapid stable attachment caused by reduced HA could allow the implantation of developmentally incompetent embryos with the potential to miscarry (Aplin *et al.*, 1996, Quenby *et al.*, 2002, Teklenburg *et al.*, 2010).

In conclusion, our study demonstrates for the first time the role of CD44 at the early stages of embryo-uterine attachment using an in-vitro implantation model and sets the scene for further investigations to determine the role of other HA-receptors and CD44 ligands at implantation.

Authors' roles

S.C.B., S.J.K., M.W. and J.D.A. designed the study, and S.J.K., D.R.B., M.W. and J.D.A. obtained funding. S.C.B. carried out the experimental work. J.D.A., S.C.B. and P.T.R. wrote the paper, which was edited by S.J.K., D.R.B. and M.W.

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Conflict of interest

None declared

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473 **Figure legends**

474 **Figure 1 CD44 in embryos and Ishikawa cells. A, B.** An E4.5 mouse blastocyst
475 fixed in PFA and stained for CD44 using polyclonal antibody H-300 (green). The

476 embryo is represented using a single Z-plane of the Z-stack together with a X-Z plane
477 image below the blue line from the point indicated by the arrow (A) or in 3D (B). **C.**
478 Rabbit serum IgG is a negative control. Blue: cell nuclei (DAPI). 5 embryos were
479 stained in 2 batches. **D, E.** Representative fluorescence images of localisation of
480 CD44 (green) at Ishikawa cell lateral membranes using monoclonal antibody 5H12 in
481 the mid-plane (D) or apical plane (E). The actin cytoskeleton is red (Alexafluor 594-
482 phalloidin). The X-Z plane (bottom of image) reveals CD44-positive epithelium. N=3.
483 **F**. Negative control (anti-KLH monoclonal with rhodamine-phalloidin and DAPI). Scale
484 bar (B, C, F) = 20μm.

485 **Figure 2 CD44 in embryo-epithelial attachment sites.** Mouse embryos attached
486 after 48 hrs co-culture with Ishikawa cells were fixed and stained with antibody 5H12
487 (green), which detects human but not mouse CD44. **A.** The main image shows
488 confluent unbreached epithelial cells. An attached embryo is centred at the position of
489 the asterisk. The X-Z plane (bottom of image) collected on the line of the arrow
490 reveals trophoblast (arrow) attached to CD44-positive epithelium. **B.** A later stage in
491 which trophoblast has displaced epithelial cells. The area lacking green staining at
492 centre reveals the position of the embryo. A blue arrow again indicates the location of
493 the X-Z section shown at bottom, with white arrows indicating the embryonic periphery
494 where trophoblast and displaced epithelium are in apposition. **C.** Still later, with
495 prominent actin bundles (arrows in top left image) characteristic of trophoblast giant
496 cells in the plane of the substrate. The position of the embryo is also revealed by the
497 absence of CD44 staining in an area left of centre. Scale bars in A, B & C, 50μm.

498 **Figure 3 Characterisation of impact of anti-CD44 antibody on embryo**
499 **attachment and stability. A, B.** Ishikawa cell spreading assay in which trypsinised

cells were plated on the indicated substrates and incubated for 1 hr. Spreading was scored with the aid of a phase contrast microscope. Control cells spread on culture plastic, poly-lysine, or osteopontin. The anti-CD44 polyclonal antibody H300 effects partial inhibition of spreading on osteopontin but does not influence behaviour on the other substrates. **C, D.** Embryo-epithelial attachment assay with H-300 antibody added just prior to co-culture from E4.5. Three conditions, control (no antibody), anti-CD44 antibody and control IgG, are respectively represented in blue, red and green. All conditions were analysed for the percentage of embryos attached either weakly, intermediately or stably, and those that had advanced to attach intermediately and stably. **E, F.** Plots show attachment when antibody was added to detached embryo co-cultures after 24h. Data are presented as mean ± SEM and statistical analysis was performed using 2-way ANOVA with Bonferroni's multiple comparison test (*: P<0.05; **: P<0.01; ****: P<0.001). N=4 (48 embryos per condition).

Figure 4 Effect of hyaluronidase treatment on embryo attachment. **A.** The HA-binding domain of versican (green) was used to reveal HA at the surface of Ishikawa cell layers. The inset shows staining in the absence of binding protein. **B.** After treatment with Hyal2, staining is lost. **C**. Quantification of HA fluorescence. **D.** CD44 staining (green) before and after Hyal2 treatment of Ishikawa cells. Total green fluorescent pixels above background before and after treatment, showing no difference. Actin, red; DNA, blue. Scale bars in A, B & D, 50μm. **E.** Quantification of CD44 fluorescence. **F**. Mouse embryo total attachment from E5.5-6.5 under four conditions of Hyal2 treatment of: embryos (red), Ishikawa cells (green), both (purple) or neither (blue). **G.** Mouse embryo stable attachment plotted. Data are presented as mean ± SEM and statistical analysis was performed using 2-way ANOVA with

524 Bonferroni's multiple comparison test (*: P<0.05; **: P<0.01; ****: P<0.001). N=4 (48
525 embryos per condition). Scale bar (A, B, D) = 50µm

Figure 1

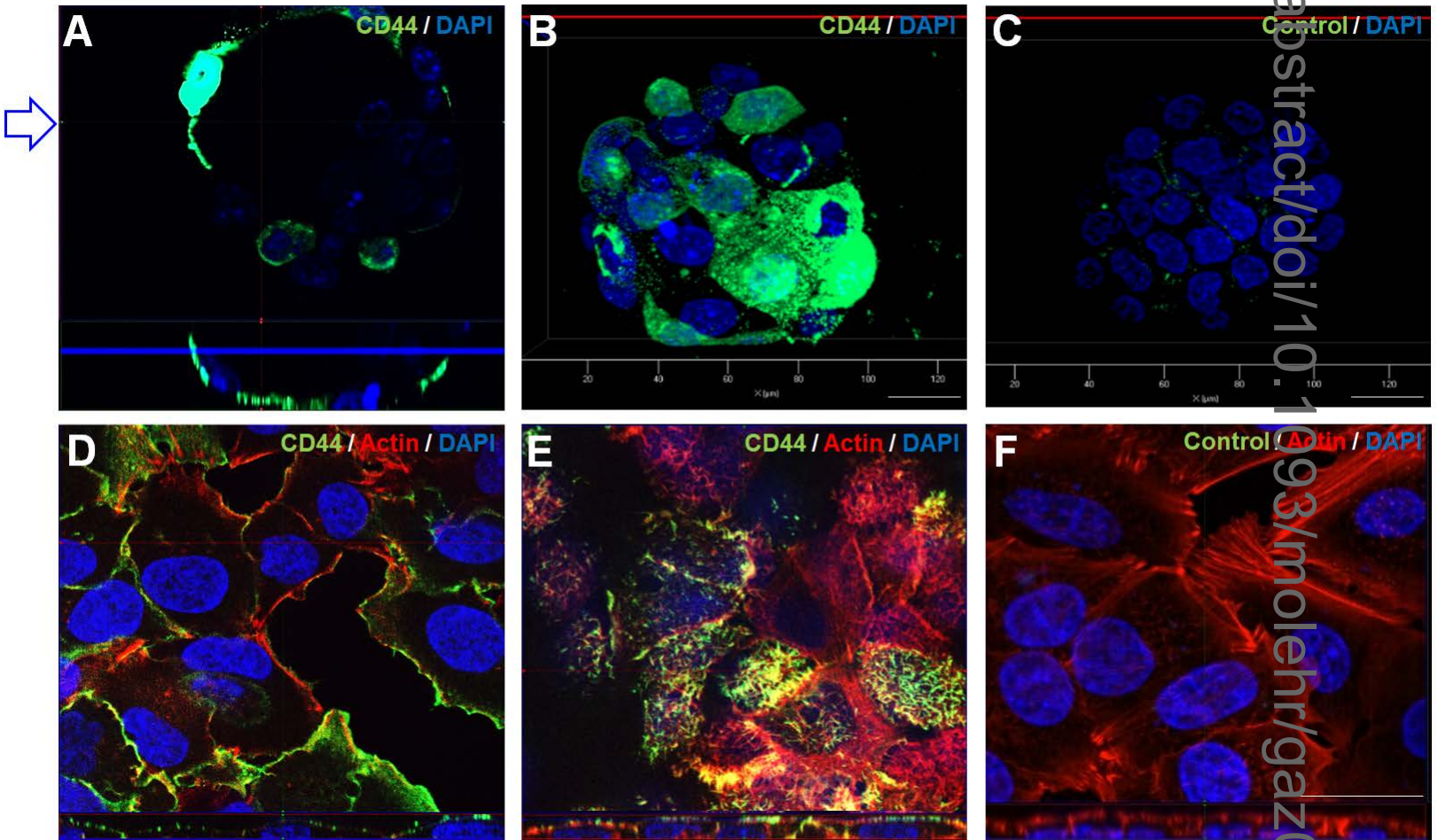

Figure 2

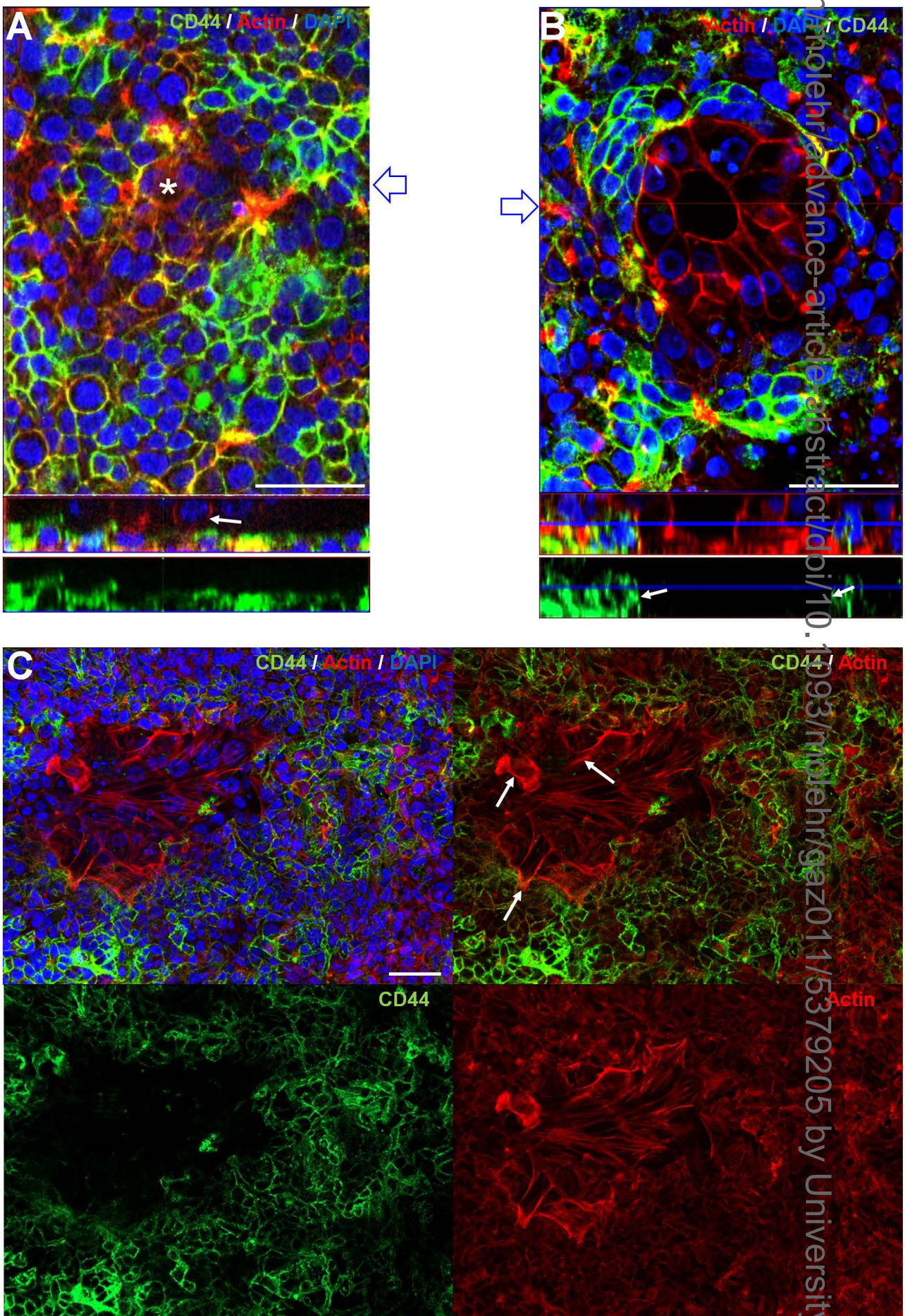

Figure 3

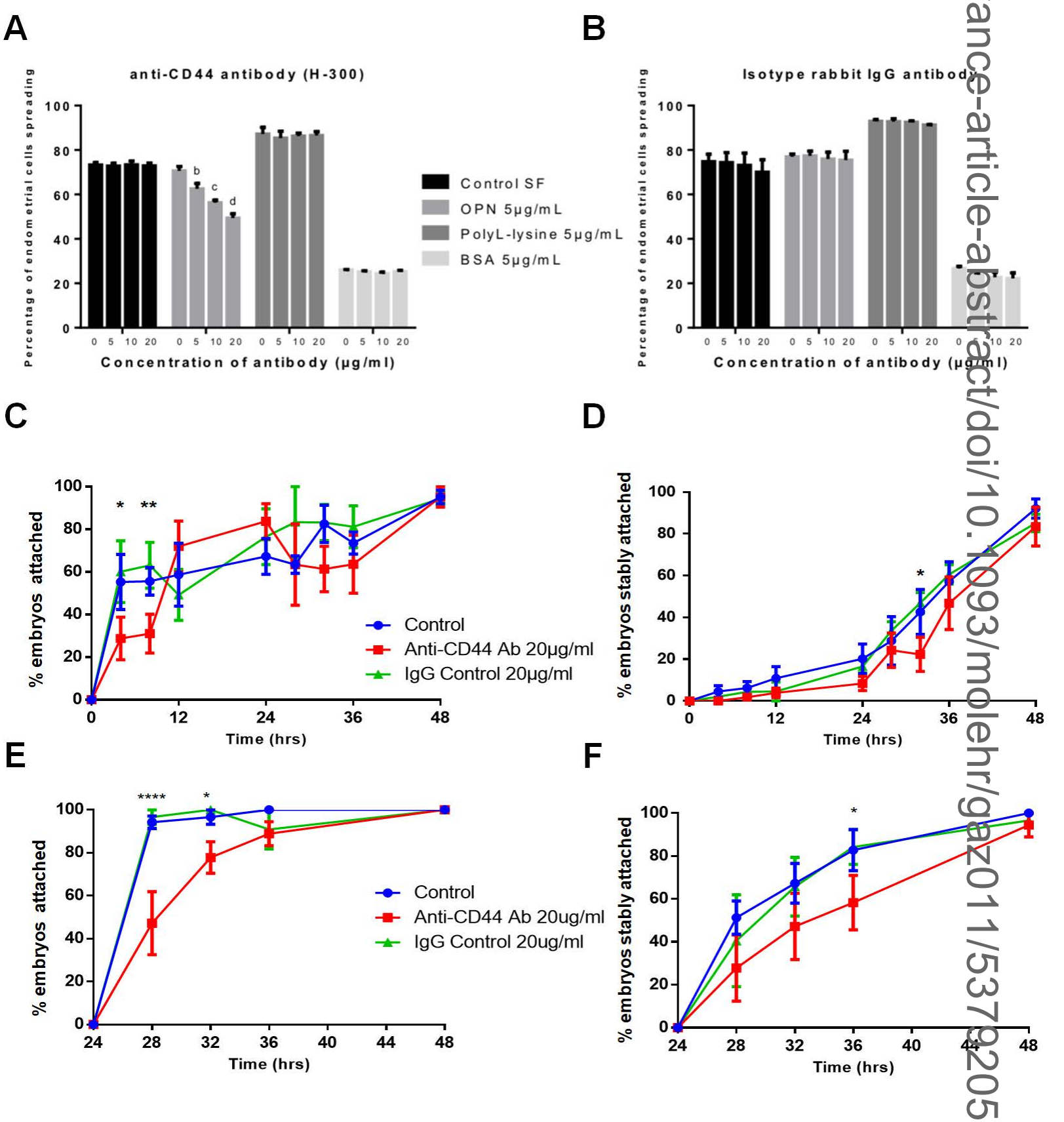

Figure 4