Histochem Cell Biol
DOI 10.1007/s00418-017-1559-3

ORIGINAL PAPER

Low molecular weight hyaluronan induces migration of human choriocarcinoma JEG-3 cells mediated by RHAMM as well as by PI3K and MAPK pathways

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Accepted: 3 March 2017

Abstract Hyaluronan (HA) is the major glycosaminoglycan present in the extracellular matrix. It is produced by some tumours and promotes proliferation, differentiation and migration among others cellular processes. Gestational trophoblastic disease (GTD) is composed by non-tumour entities, such as hydatidiform mole (HM), which is the most common type of GTD and also malignant entities such as choriocarcinoma (CC) and placental site trophoblastic tumour (PSTT), being CC the most aggressive tumour. Although there is a growing understanding of GTD biology, the role of HA in the pathogenesis of this group of diseases remains largely unknown. The aim of this work was to study the role of HA in the pathogenesis of GTD by defining the expression pattern of HA and its receptors CD44 and RHAMM, as well as to determine if HA can modulate proliferation, differentiation and migration of CC cells. Receptors and signalling pathways involved were also analyzed. We demonstrated that HA and RHAMM are differently expressed among GTD entities and even among trophoblast subtypes. We also showed that HA is able to enhance the expression of extravillous trophoblast markers and also to induce migration of JEG-3 cells, the latter mediated by RHAMM as well as PI3K and MAPK pathways. These findings indicate a novel regulatory mechanism for CC cell biology and also contribute to the understanding of GTD pathophysiology.

Keywords Hyaluronan · RHAMM · Hydatidiform mole · Choriocarcinoma · Migration · Signalling pathways

Introduction

The extracellular matrix (ECM) is a complex mixture of proteins, glycosaminoglycans and proteoglycans that play an active role in physiological and malignant contexts.

Hyaluronan (HA) is the major glycosaminoglycan present in the ECM. It comprises *N*-acetyl-glucosamine and *N*-acetyl-glucuronic acid. HA levels result from a balance between its synthesis mediated by HA synthases (HAS1-3) (Weigel and Deangelis 2007), its internalization by receptors and its degradation by hyaluronidases (HYAL1-3) (Stern and Jedrzejas 2006). HA is associated with many important cellular processes such as proliferation, wound healing, inflammation, hydration, migration and invasion, not only during physiological but also in processes involving transformed malignant cells. It has also been reported that HA participates in multidrug resistance, evasion of apoptosis, senescence and epithelial-mesenchymal transition (Alaniz et al. 2006; Cordo Russo et al. 2010; Lompardía et al. 2013; Sironen et al. 2011; Toole 2009). These functions are mediated by interaction with several receptors

Electronic supplementary material The online version of this article (doi:10.1007/s00418-017-1559-3) contains supplementary material, which is available to authorized users.

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Published online: 01 April 2017

such as CD44, RHAMM, TLR-2/4, Lyve and HARE, being CD44 and RHAMM the main receptors involved in HA-mediated processes in malignant context (Jiang et al. 2011).

CD44 is the most known and studied HA receptor. It is a transmembrane protein whose extracellular membrane portion size can vary depending on the splicing of its original mRNA, also affecting its ability of HA binding. While the external portion function is to bind ligands, the internal portion transduces signals into the cytoplasm (Naor 2016; Toole 2009; Turley et al. 2001).

Receptor hyaluronan-mediated motility (RHAMM) is an itinerant protein which can be present in the outer side of the cell membrane, in the cytoplasm or even in the nucleus (Assmann et al. 1998; Wang et al. 1998; Zhang et al. 1998). RHAMM has not a transmembrane domain and partners with other proteins such as CD44 and EGF among others (Maxwell et al. 2008; Turley et al. 2012, 2016).

It has been previously demonstrated that HA-CD44 and HA-RHAMM interactions are able to trigger different signalling pathways such as PI3K/Akt and MAPK, which are involved in proliferation, differentiation and migration (Cordo Russo et al. 2010; Cordo Russo et al. 2012; Lompardía et al. 2013; Monslow et al. 2015). However, the implication of HA in the pathophysiology of gestational related neoplasia have been poorly studied so far.

Gestational trophoblastic disease (GTD) comprises a group of trophoblast-related disorders classified as hydatidiform moles [partial (PHM) or complete (CHM)], and trophoblastic tumours [as choriocarcinoma (CC) and placental site trophoblastic tumour (PSTT)] (Shih 2007).

Hydatidiform mole is an abnormal pregnancy whose clinical course can vary: it may evolve to spontaneous regression, quiescent trophoblastic disease or progression to a gestational trophoblastic tumour (GTT). For example, the probability of appearance of CC increases particularly after a complete mole rather than after a partial one (Mangili et al. 2014).

Hydatidiform mole (and also normal pregnancy) is composed of villous cytotrophoblast (CT), syncytiotrophoblast (ST) and intermediate trophoblast (IT). They can be distinguished morphologically but also through its characteristic trophoblast markers. IT expresses HLA-G (Singer et al. 2002) while ST expresses Syncytin-2 (Vargas et al. 2009) and is able to secrete hCG (Cole 2010). The previously mentioned three cell types arise from a trophoblastic stem cell and are also present in GTT as was demonstrated by immunohistochemical studies, being the implantation site and chorionic-type intermediate trophoblast prevalent in CC and PSTT, respectively (Mao et al. 2007).

Trophoblastic tumours are able to metastasize to organs such as lung, gastrointestinal tract, spleen, kidney, liver and brain. Although CC is a highly malignant neoplasm, it is more sensitive to chemotherapy than PSTT even with extrauterine spread. Meanwhile hysterectomy is the main therapeutic approach for PSTT even when disease is limited to the uterus (Sebire and Lindsay 2010).

Although epidemiological data about GTD scarce, it has been reported an incidence of HM from 1.5–6 per 1000 pregnancies in South America while CC may occur in 2–7 per 100,000 pregnancies and the frequency of PSTT was estimated to be 0.2% of all GTD (Mangili et al. 2014).

All these diseases increase probabilities of complications or even death in women, and due to the limited number of tissue specimens and animal models available many biological and molecular aspects of the biology of GTD are still poorly understood, including those related to the ECM.

The aim of this work was to evaluate the expression and distribution of HA, RHAMM and CD44 in GTD tissues as well as to study the biological and molecular mechanisms induced by HA. Our results show that HMW- and LMW-HA induces cell differentiation into a migratory phenotype but do not affect cell proliferation. However, only LMW-HA is able to induce cell migration. This effect was mediated by RHAMM as well as PI3K/Akt and MAPK pathways. To our knowledge this is the first report demonstrating RHAMM expression as well as the role of its interaction with HA in choriocarcinoma.

Materials and methods

Reagents

Recombinant high (HMW 1.5–1.8 × 106 Da) and low molecular weight HA (CPN spol.s.r.o Czech Republic) was supplied by Farmatrade (Argentina). Anti-CD44 monoclonal Ab (MAb) (IM7) was purchased from ATCC (USA). HYAS bovine testes (#H3884, Type IV-S, lyophilized powder, essentially salt-free), UO126 and LY294002 were purchased from Sigma–Aldrich (USA). Biotinylated HA-binding protein (bHABP, #385911) was purchased from Calbiochem (USA). Abs against pERK, ERK, β-Actin, RHAMM, anti-rat secondary horseradish peroxidase, anti-rabbit secondary horseradish peroxidase, anti-goat secondary horseradish peroxidase, bioltinylated anti-rat secondary and biotinylated anti-goat secondary Abs were purchased from Santa Cruz Biotechnology (USA). [^{3}H]-thymidine was purchased from Perkin-Elmer (Boston, USA). RPMI-1640, DMEM, Ham, L-glutamine, streptomycin and penicillin were purchased from Invitrogen (Argentina).

Tissue collection

A total of 13 paraffin-embedded tissues provided from the Pathology Service of Hospital General de Agudos Carlos G Durand, Buenos Aires, Argentina, were used for the study.

Samples were obtained from first-trimester complete hydatidiform mole (CHM $n=5$), choriocarcinoma (CC $n=2$), placental-site trophoblastic tumour (PSTT $n=2$) and first-trimester placenta (control $n=4$). Tissues were selected for immunohistochemical studies after hematoxylin and eosin slides were reviewed. The diagnosis of CHM was established before the evacuation time according to biochemical (human chorionic gonadotrophin, hCG value), echographic and clinical parameters and then confirmed by histological evaluation. Clinical information and tissue samples were obtained under the approval of the Ethics Committee of Hospital General de Agudos Carlos G. Durand.

Histochemistry for hyaluronan

Hyaluronan staining was performed as described previously (Cordo Russo et al. 2012). After deparaffination, to block endogenous peroxidase activity slides were incubated with 3% H_2O_2 in methanol for 15 min at room temperature (rt). To block non-specific binding sites, sections were incubated with 10% fetal bovine serum in PBS for 30 min at rt, followed by an avidin and biotin blocking solution (Vector, Peterborough, UK). Then, biotinylated-hyaluronan binding protein (bHABP) (Calbiochem, Darmstadt, Germany) was added and incubated overnight. As amplification and revealing system, an avidin-peroxidase complex (Vector, Peterborough, UK) was used. The reaction product was visualized by the addition of a diaminobenzidine solution (DAB) (Vector, Peterborough, UK) followed by counterstaining with Mayer's hematoxylin. To assess non-specific binding of bHABP tissue sections were either stained with bHABP that had been pretreated with 50 U/ml Hyaluronidase form bovine testes (Sigma, USA) in PBS at 4 °C overnight or incubated without the addition of bHABP.

Immunohistochemistry for RHAMM and CD44

After deparaffination, a heat retrieval antigen step was performed. Blocking endogenous peroxidase activity and non-specific binding sites steps were carried out as described above. Slides were then incubated with 1:50 dil of RHAMM polyclonal antibody (E-19, sc-16170, Santa Cruz Biotechnology, Santa Cruz, USA) or 1:25 dil of CD44 monoclonal antibody (IM7, ATCC, USA) overnight at 4 °C. Slides were washed and incubated with anti-goat IgG-HRP and anti-rat F(ab)'2-HRP (Santa Cruz Biotechnology, Santa Cruz, USA) 2 h and overnight at 4 °C at rt, respectively. Slides were washed again and the reaction product was visualized by adding a diaminobenzidine (DAB) solution (Vector, Peterborough, UK) followed by counterstaining with Mayer's hematoxylin. For negative controls, no primary antibody was added. For positive controls, prostate and colorectal cancer tissues were used for RHAMM and CD44, respectively (Gust et al. 2009; Lugli et al. 2010).

Cell culture

Human choriocarcinoma cell lines, BeWo, JEG-3 and JAR (gently donated by Dr Susana Genti Raimondi, Córdoba, Argentina) were used. JEG-3 and JAR were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 100 U/ml penicillin and streptomycin. In similar conditions, BeWo was maintained in Ham medium. HaCaT cells were used as positive control of hyaluronan-metabolizing enzymes (Averbeck et al. 2006).

Measurement of HA levels by enzyme-linked inmunoabsorbent assay

Supernatants were taken from a monolayer 50% confluent in a 6-well plate and HA levels were measured by a competitive ELISA-like assay as described previously (Lompardía et al. 2013). Briefly, 96 well microtiter plates were coated with 100 µg/mL HMW-HA at 4 °C. Samples and standard HMW-HA were incubated with 0.75 µg/mL bHABP at 37 °C. The plate was blocked and incubated with the samples at 37 °C for 4 h. The bHABP bound was determined using an avidin–biotin detection system. Sample concentrations were calculated from a standard curve.

Reverse-transcription polymerase chain reaction (RT-PCR)

The reaction was carried out as was described previously (Cordo Russo et al. 2012). Cells were cultured until 50% confluence in a 6-well plate. Then cells were treated with 1 ml TRIZOL (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. Isolated RNA was reverse-transcripted and cDNA was then amplified with specific primers for human HAS2, HAS3, Hyal1 and Hyal2 (see Table 1). Thirty-five PCR cycles were carried out with an appropriate annealing temperature for every product and followed by a 5 min extension at 72 °C. To ensure that load was kept constant in all lanes, beta actin and gpdh genes were used as amplification control. PCR products were separated by agarose gel electrophoresis (2,5%) and stained with Gel Red (Biotium). Gel images obtained with a UV transilluminator (Cole Palmer Instrumental, IL, USA) and a digital camera were subjected to densitometric analysis using Image Scion Software (Scion Corporation, USA).

Table 1 Primer sequences used for RT-PCR

Gene	Primer sequence (Fw)	Primer sequence (Rv)	Ta (°C)	bp
CD44	AAGACATCTACCCCAGCAAC	CCAAGATGATCAGCCATTCTGG	52	324
RHAMM	TCCTAAGGCGCCCTTGAAAC	TGCAGCATTTAGCCTTGCTT		v1 361 v2 358 v3 313
HAS2	GCCGGTCGTCTCAAAATC	TCTCACAATGCATCTTGTTCAGC	55	133
HAS3	CTCTACTCCCTCCTCTATATGTC	AACTGCCACCCAGATGGA	50.7	150
Hyal1	AAGCCCTCCTCCTCCTTAACC	AGCCAGGGTAGCATCGAC	55	141
Hyal2	TTGTGAGCTTCCGTGTTCAG	GTCTCCGTGCTTGTGGTGTA	53.3	217
HLA-G	GCTACTCTCAGGCTGCAATGTGAA	CACAGGGGTGGGCTGGTCTCT	57	115
Sincityn-2	ATCCAGCCTCGCCCAGAGAATGG	GCTTGGGCGTCCCTGGCAAAA	58	422
Beta actin	AGCCTCGCCTTTGCCGATCC	ACCATCACGCCCTGGTGCCT	60	185
GAPDH	CCAGCCGAGCCACATCGCTC	ATGAGCCCCAGCCTTCTCCAT	60	360

Ta anneling temperature, *bp* base pair

Indirect immunofluorescence

JEG-3 cells were cultured on coverslips until 50% confluence in a 24-well plate and IF was carried out as previously described, with few modifications (Lompardía et al. 2013). Cells were blocked with PBS containing 2% normal human serum, fixed with PBS plus 4% PFA (*p*-formaldehyde) for 15 min and washed. Then cells were incubated with anti RHAMM Ab, followed by a FITC-conjugated secondary Ab and DAPI. Cells were analyzed by an Olympus BX51 (Olympus Corporation) fluorescence microscopy.

Cell proliferation

Cell proliferation was analyzed by [^{3}H]-thymidine incorporation assay evaluated at 48–72 h in 96-microtiter well plates as described previously (Lompardía et al. 2016). Five thousand cells per well were used. Cells were grown at 37 °C in a 5% CO_2 atmosphere with RPMI, 62,5-500ug/ml HMW- or LMW-HA. After pulsing with 1uCi [^{3}H]-thymidine for 6 h, cells were harvested and counted in a liquid scintillation counter (Beckman, MD). Results were calculated from mean cpm of [^{3}H]-thymidine incorporated in quadruplicated cultures. Untreated cells represented 100% cell survival.

Wound-healing assay

Cells were seeded in 24-wells plate until confluence. Monolayer was cut with a 200 ul sterile pipette tip and cells were incubated with different concentrations of several molecular weight HA as indicated above. Photographs were taken at 0 and 24 h at the same position. Results were expressed as migration index calculated as: $[(100-\%\ \text{area})_{t=24\ \text{h}}-(100-\%\ \text{area})_{t=0\ \text{h}}]_{\ \text{treated}}/[(100-\%\ \text{area})_{t=24\ \text{h}}-(100-\%\ \text{area})_{t=0\ \text{h}}]_{\text{untreated}}$. T-scratch software was used to calculate acellular area (Gebäck et al. 2009).

Chemotaxis assay

Chemotaxis assay was performed as previously described with a few modifications (Cordo-Russo et al. 2010; Ernst et al. 2014). Migration was measured using a 24-transwell plate (8 µm pore size, BD FalconTM, USA). Cells (1.5×10^5) were placed in the upper chamber of the transwell unit while the lower wells contained LMW-HA or RPMI 1640. After incubation for 24 h at 37 °C, the membrane was removed and cells on the upper side of the membrane were removed using cotton swabs. Cells attached to the lower side of the membrane were fixed in 2% formaldehyde and stained in 10% DAPI. Cells were counted in a fluorescence microscope (OLYMPUS BX51, Olympus Corporation) using a 40× objective. Ten fields per well were recorded and the results were presented as percentage of cell migration relative to control. To evaluate the capacity of RHAMM to modulate HA-mediated migration, cells were treated with anti-human RHAMM polyclonal antibodies (10ug/ml) or with normal goat IgG (isotype control) for 30 min at 4 °C before migration assay. To evaluate the capacity of endogenous hCG to modulate HA-mediated migration, cells were treated with anti-human hCG monoclonal antibodies (10 and 20ug/ml) or with normal mouse IgG2κ (isotype control) for 30 min at 4 °C before migration assay. Besides, the ability of PI3K and MAPK inhibitors to modulate HA-mediated migration was tested incubating cells with LY294002 (2 µM) and UO126 (4 µM) or with DMSO (control) for 30 min at 4 °C before migration assay. To evaluate random migration, chemokinesis controls were performed by adding HA to both, the upper and lower compartment of the chamber.

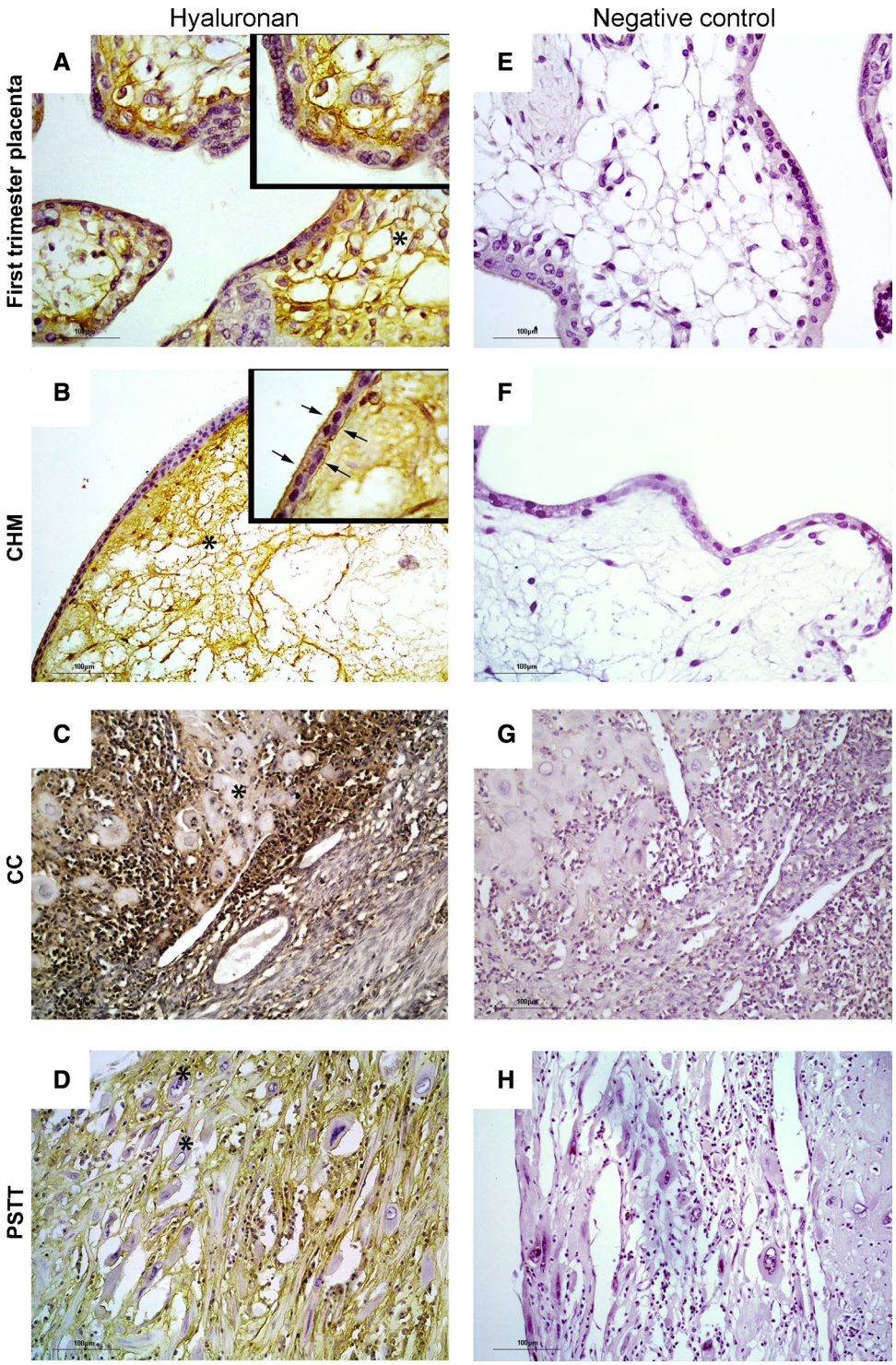

Fig. 1 HA histochemistry. **A** First trimester placenta. Stromal villous expression was positive. Instead, no expression on membranes was found (*asterisk*). **B** CHM. Stromal villous expression was positive (*asterisk*) as well as *apical* and *basal* membrane of villi (*arrow*). **C** CC and **D** PSTT: tumour stroma was positive while no HA expression was found on tumour cells (*asterisk*). **E–F** Negative control. *Scale bar* represents 100 µm

Immunoblotting

Cells were treated with RPMI, 500ug/ml HA-LMW, 2 µM LY294002, 4 µM UO126 and 10ug/ml RHAMM Ab or combination for 2 or 24h at 37 °C in a 5% CO_2 atmosphere. Cells were then lysed with hypotonic buffer. After centrifugation, equal amount of protein were resolved by SDS–polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Osmonics Inc, Gloucester, MA). The membrane was blocked and incubated with specific antibodies to CD44, RHAMM, Akt, pAkt, ERK, pERK or beta-actin overnight at 4 °C followed by incubation with horseradish peroxidase-labelled secondary antibody for 2 h at 37 °C. The detection system and densitometric analysis

Table 2 Location of HA in GTD entities and first trimester placenta

	Trophoblast cell	Villous cell surface	Villous stroma	Non-villous stroma
First trimester placenta	−	−	+	−
CHM	−	+*	+	
CC	−			+
PSTT	−			+

CHM complete hydatidiform mole, *CC* choriocarcinoma, *PSTT* placental site trophoblastic tumour

+ Present, − Absent

*Some areas showed simultaneous staining in apical and basal membrane

was performed as described previously (Lompardía et al. 2016).

Statistical analysis

Statistical significance between groups was evaluated by one way-ANOVA and means were compared by the Dunnet's test (wound-healing and proliferation assays) or Bonferroni's test (ELISA, transwell assays and densitometric analysis). Differences between groups were considered significant at the level of $P < 0.05$. Analysis was performed using Prism 4 software (Graph Pad, San Diego, CA, USA).

Results

HA and RHAMM but not CD44 are expressed in trophoblastic tissues

First, we studied the expression and distribution of HA, RHAMM and CD44 on human biopsies of trophoblastic diseases. The expression of hyaluronan was evaluated by histochemical studies while the expression of RHAMM and CD44 were analysed by immunohistochemistry.

As shown in Fig. 1, HA was expressed in all samples analysed. HM was classified as CHM after p57 staining (Fig S1). In first-trimester placenta, positive staining for HA in the villous stroma was detected (Fig. 1A). CHM also showed HA staining in the villous stroma (Fig. 1B) but in a different pattern from that of controls (placenta), showing a compact fibrilar appearance compared with first-trimester placenta. Interestingly, HA was expressed in isolated tissue areas where the apical and basal membranes of villi were stained simultaneously (Fig. 1A, inset) while it was not expressed in villous membranes of first-trimester placenta (Fig. 1B, inset). In trophoblastic tumours, intense positive expression of hyaluronan was detected in tumour stroma, whereas no staining was found in tumour cells from choriocarcinoma or PSTT (Fig. 1C, D, respectively). HA expression among GTD entities is summarized in Table 2.

RHAMM immunoreactivity was found in the cytoplasm of the villous cytotrophoblast and on the cell surface of intermediate trophoblast in first-trimester placenta (Fig. 2A, B) and CHM (Fig. 2C). In trophoblastic tumours, RHAMM staining was found on the cell surface in choriocarcinoma (Fig. 2D) and in the nucleus of some cells of PSTT (Fig. 2E). Different expression patterns of RHAMM among trophoblast subpopulations in GTD entities are summarized in Table 3. A positive immunostaining for the CD44 receptor was found in CC tumour stroma but not in tumour cells. Instead, CD44 expression was absent in first-trimester placenta, CHM or PSTT tumour cells (Fig. 3).

Human CC cell lines express differently HA, HA-metabolizing enzymes and RHAMM

To further investigate the role of HA and RHAMM in choriocarcinoma biology and to select the best model of study we characterized the human CC cell lines JAR, JEG-3 and BeWo. We evaluated HA secretion by ELISA, HA-metabolizing enzymes expression by RT-PCR as well as RHAMM and CD44 expression by RT-PCR and IIF.

Our results showed that BeWo but not JAR and JEG-3 cells secreted HA to its extracellular media when compared with culture media alone (Fig. 4A). Besides, all cell lines expressed mRNA of HA-metabolizing enzymes HAS3, Hyal1 and Hyal2 but no HAS2 (Fig. 4B). Further studies on the activity of HAS as well as Hyal would provide more information about the amount of HA showed in this study since mRNA levels could not explain this directly. Moreover, although all cell lines expressed three mRNA RHAMM variants (Fig. 4C), it is JEG-3 cell line which shows higher RHAMM protein expression than JAR and BeWo cells (Fig. 4D). This may be due because the final translation of such RNA could not reflect the protein expression level. None of the three lines express CD44 neither at mRNA or protein level, as was described previously by other authors (data not shown) (Takahashi et al. 2014).

Considering the results obtained for HA, RHAMM and CD44 expression in human tissues and in vitro models of choriocarcinoma we selected JEG-3 cell line as the best model to study the role of such molecules in this disease.

LMW-HA but no HMW-HA induces dose-dependent JEG-3 cell migration

Taking into account that CC is a highly invasive disease and also that HA and RHAMM have been involved in migration and proliferation processes in others types of

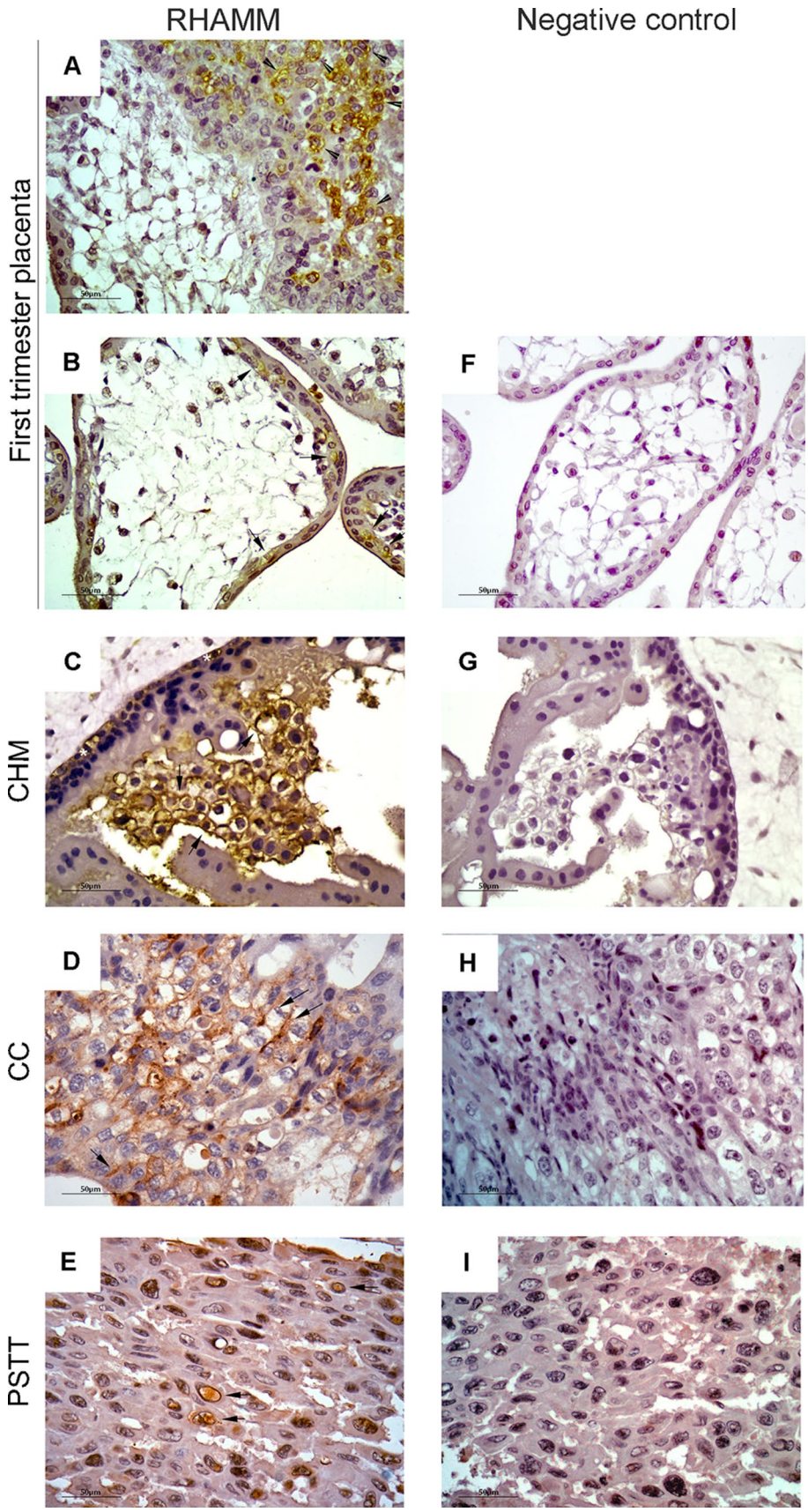

Fig. 2 RHAMM immunohistochemistry. First trimester placenta. **A** RHAMM expression was found in cell surface of IT (*arrowahead*) and **B** in cytoplasm of CT cells (*arrow*). **C** CHM. RHAMM immunoreactivity was found in cell surface of IT (*arrow*) and cytoplasm of CT cells (*white asterisk*). **D** CC. RHAMM expression was present in cell surface of tumour cells (*arrow*). **E** PSTT. Nuclear expression was positive in some tumour cells (*arrow*). **F–J** Negative control. *Scale bar* represents 50 µm

Table 3 Location of RHAMM in GTD entities and first trimester placenta

	Trophoblast subtype	Expression pattern			
		Cell surface	Cytoplasm	Nucleus	Stromal cell
First trimester placenta	CT	−	+	−	−
	IT*	+	+	−	
	ST	−	−	−	
CHM	CT*	+	+	−	+
	IT	+	−	−	
	ST	−	−	−	
CC	IT	+	−	−	
	ST	−	−	−	
PSTT		−	−	+	

CT cytotrophoblast, *IT* intermediate trophoblast, *ST* syncytiotrophoblast, *CHM* complete hydatidiform mole, *CC* choriocarcinoma, *PSTT* placental site trophoblastic tumour

+ Present, − Absent

*Some cells shows simultaneous location staining

cancer, we first studied the effect of different concentrations of LMW- and HMW-HA on such cellular processes.

As is shown in Fig. 5A, B, we found that LMW-HA (but no HMW-HA) induced JEG-3 cell migration in a dose-dependent manner by wound healing assay. Then, to ratify this result we performed a chemotaxis assay and we found that 500 µg/ml LMW-HA is able to induce cell migration. This also suggests that directional migration is due to either chemotaxis or haptotaxis (Fig. 5C). In addition, to corroborate that the above results were not due to an increase in cell number we studied the effect of different doses of LMW- and HMW-HA on cell proliferation by [^{3}H]-thymidine incorporation assay (Fig. 5D). We found that HMW-HA exerted no effect on such process while low doses of LMW-HA were able to reduce proliferation. Importantly the highest dose of LMW-HA did not affect proliferation which ratified the effect of LMW-HA on cell migration.

HA enhances the expression of extravillous trophoblastic markers

We also asked whether HA could modulate the expression of trophoblastic markers and induce a particular phenotype. To achieve this aim we evaluated the mRNA expression of HLA-G and Syncytin-2 by RT-PCR as well as the secretion of hCG by ECLIA.

We found that both LMW- and HMW-HA induced an increase of mRNA expression of HLA-G (Fig. 6A) and a decrease of mRNA expression of Syncytin-2 (Fig. 6B) and hCG secretion (Fig. 6C). These results indicated that both molecular weights of HA modulate the expression of such trophoblastic markers into an extravillous trophoblast phenotype which is responsible of migration and invasion into the maternal tissues. This also supports the above results which showed that LMW-HA induced JEG-3 cell migration.

PI3K and MEK are involved in LMW-HA-induced migration

The main activated signaling pathways under microenvironment stimulus, as HA, are MEK/ERK and PI3K/Akt. Taking this into account, we then studied the involvement of PI3K and MEK kinases in the effect exerted by HA on JEG-3 cell migration.

Using a pharmacological approach we showed by chemotaxis assay that the inhibition of PI3K but not MEK activity was able to impair the effect of HA on cell migration (Fig. 7A-I). To go into deep we also evaluated the pERK/ERK and pAkt/Akt ratio by western blot. Unexpectedly we failed to demonstrate pAkt expression in the evaluated conditions which do not let us achieve to a pAkt/Akt ratio (data not shown). Instead, we found that HA enhanced pERK/ERK ratio but it was not able to reverse the effect of both inhibitors (U0126 and Ly249002) when they were administered in combination with HA (Fig. 7A-II). These results indicate that HA induces cell migration through PI3K and also that a crosstalk between the pathways govern by this kinase and MEK may occur.

RHAMM modulates LMW-HA-induced migration through ERK activation

Finally, we hypothesized that RHAMM may be implicated in the migration induced by LMW-HA. To evaluate this, we used a blocking Ab against RHAMM and then performed chemotaxis assay. We found that after blocking with RHAMM antibody, cell migration was decreased and it could not be reversed by addition of HA (Fig. 7B-I). These results demonstrate that RHAMM was able to affect both HA-independent as well as HA-dependent cell migration.

We also analyzed the expression of pERK showing that HA was not able to reverse the blocking effect of RHAMM (Fig. 7B-II). As expected, we found that pERK is under HA-RHAMM signalling in the control of migration.

Discussion

Gestational trophoblastic diseases are rare condition malignancies and have a highly curable rate after an accurate diagnostic, therapeutic and follow-up management (Sebire 2010; Sebire and Lindsay 2010). However,

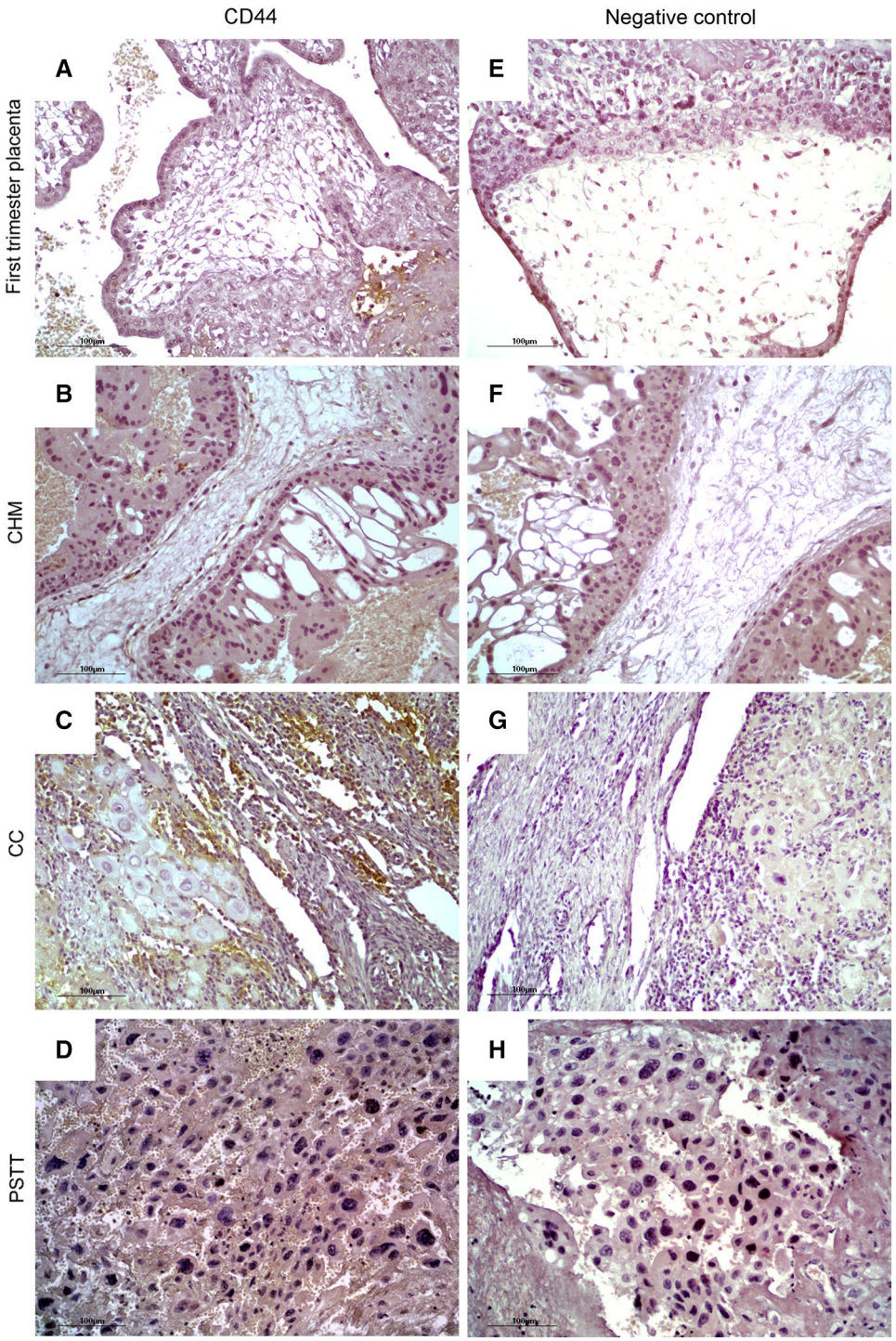

Fig. 3 CD44 immunohistochemistry. In **A** first trimester placenta, **B** CHM and **D** PSTT: CD44 expression was absent. **C** CC. Stromal CD44 staining was found. **E**–**H** Negative control. *Scale bar* represents 50 µm

it is still needed to understand the biology of such diseases to achieve a potential therapeutic target for those resistant and recurrent cases. It is known that an important crosstalk between cells and surrounding extracellular matrix components favour multiple cellular processes. This knowledge had led to look for new targets related with the ECM.

The role of HA and its receptors RHAMM and CD44 in solid (Sironen et al. 2011) and onco-hematological (Zoller 2015) tumours had been studied while its involvement on gestational related neoplasias remains poorly understood.

In this work, we demonstrated the expression pattern of HA, RHAMM and CD44 in GTD tissues. We also studied the role of HA in CC cell proliferation, differentiation and

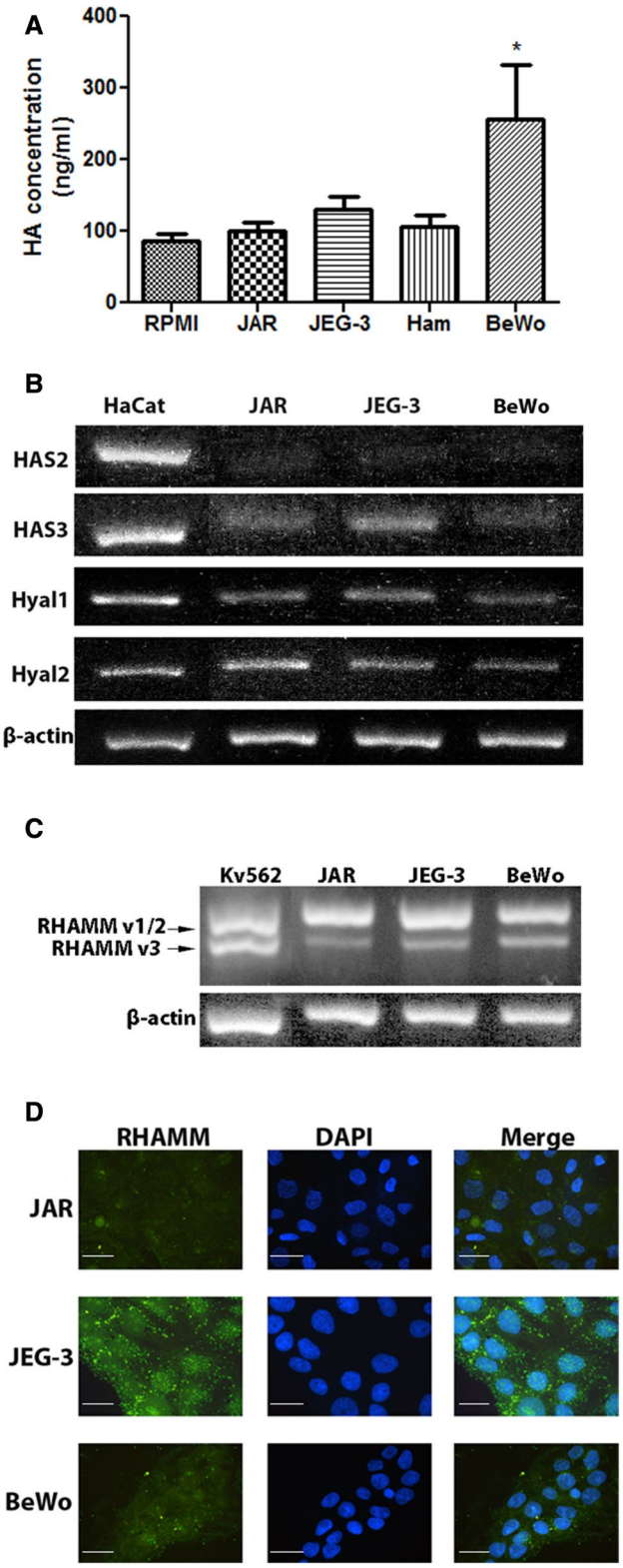

Fig. 4 Human CC cell lines characterization. **A** HA secretion evaluated by ELISA. Values are expressed as the mean ± SEM of HA concentration (ng/mL) of at least three independent experiments $*P < 0.05$ (BeWo compared to Ham). **B** mRNA expression of HAS (HAS-2 and HAS-3) and Hyal (Hyal-1 and Hyal-2) was evaluated by RT-PCR. β-actin was used as control of near equal amplification. HaCaT cells were used as positive control. **C** mRNA expression of RHAMM was evaluated by RT-PCR. β-actin was used as control of near equal amplification. Kv562 cells were used as positive control. **D** RHAMM expression on cell surface evaluated by IIF. *Scale bar* represents 50 µm

migration as well as the underlying mechanisms involved on such processes.

We found that in CHM HA is present in villous stroma as well as in the apical and basal membranes of villi whereas no expression in membranes was found in first-trimester placenta. In CC and PSTT, we found HA expression related to tumour stroma but not in malignant cells. Our results are partly according with those from Goshen et al. who described HA expression in villous stroma in CHM as well as its depletion in choriocarcinoma cells in a few specimens of GTD tissues (Goshen et al. 1996). Also our results suggest that HA metabolism may be altered in trophoblast cells from both CHM and trophoblastic tumours.

We also studied the HA receptors expression. In CHM, we failed to find CD44 expression in trophoblast cells in accordance with other authors (Reynolds et al. 2007). It was previously reported a down-regulation of mRNA and protein expression of CD44 in CHM as well as no association between CD44 expression and progression to gestational trophoblastic neoplasia after CHM (Kato et al. 2002; Reynolds et al. 2007). In trophoblastic tumours, we only found CD44 expression associated to tumour stroma in choriocarcinoma but not in malignant cells.

To our knowledge, this is the first report demonstrating the protein expression and localization of RHAMM in early placenta and GTD tissues. Besides, RHAMM mRNA expression in the placenta as well as up-regulation in CHM has been previously reported (Greiner et al. 2002; Kato et al. 2002). Also up-regulation of RHAMM in human trophoectoderm cells during the implantation window has been described (Jiang et al. 2013), suggesting a temporal expression of this protein. In a different development time as embryo stage, RHAMM expression has also been found in both human and murine species where its downregulation led to a reduced pluripotency of embryonic stem cells promoting differentiation of these cells (Choudhary et al. 2007; Jiang et al. 2013). In the present study, we showed cell surface expression of RHAMM in intermediate trophoblast, which is related to migration and invasiveness, suggesting a relevant function promoting cell migration. Moreover, expression of RHAMM into the cytoplasm of CT but its absence in ST together with the above findings in IT suggests that this protein may suffer post-translational modifications during trophoblast differentiation. All this together let us suggest that RHAMM variants might be expressed in GTD entities leading to a differential

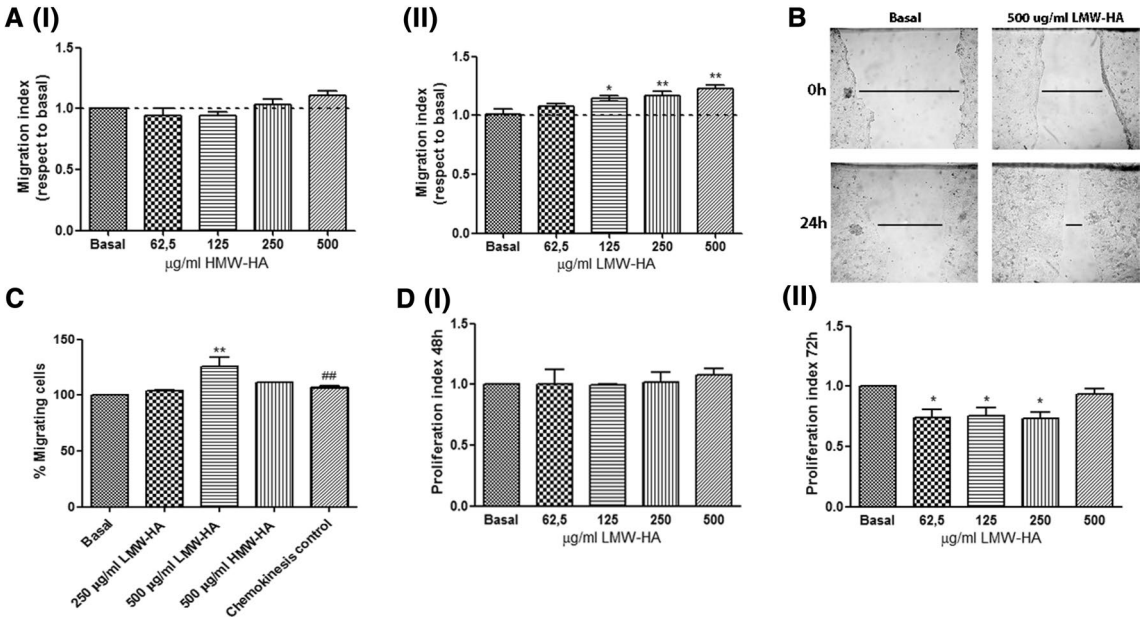

Fig. 5 Effect of HA on cell migration and proliferation. **A** JEG-3 cell migration was evaluated after treatment with a range (62.5–500 µg/ml) of HA concentration of HMW-HA (I) and LMW-HA (II) for 24h by wound healing assay. Control cells were untreated. **B** Representative image of an acellular area in HA treated and control conditions at 0 and 24h. **C** JEG-3 cell migration was evaluated after treatment with 250 and 500 µg/ml LMW-HA and 500 µg/ml HMW-HA by chemotaxis assay. Control cells were untreated. The chemokinesis control was performed by adding LMW-HA (500 µg/ml) to both, the upper and the lower compartment of the chamber. **D** JEG-3 cell proliferation was evaluated after treatment with LMW-HA during 48h (I) and 72h (II). Control cells were untreated. The results are expressed as relative index compared with control. *Bars* represent the mean ± SEM of at least three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ (respect to *basal*). $^{\#\#}P < 0.01$ (respect to 500 µg/ml LMW-HA treatment)

localization of RHAMM, which might be related with the different ability of such entities to migrate to distant organs.

Further, we characterized three human CC cell lines BeWo, JEG-3 and JAR. We described for the first time that CC cell lines secrete differently HA into the culture media as well as express mRNA of HA-metabolizing enzymes. Only BeWo cells secretes HA into the culture media although the three cell lines express mRNA of HAS3, Hyal1 and Hyal2 but not for HAS2. It was reported that first-trimester trophoblast expresses mRNA of HAS2 but not of HAS1 or HAS3 (Zhu et al. 2013). While our results let us to hypothesize that an altered metabolism of HA (which can also affect the MW-HA secreted) may be present in choriocarcinoma, a detailed study of the activity of HAS and Hyals will give more insight. Instead, mRNA or protein levels could not be representative.

We showed for the first time the expression of at least two variants for RHAMM mRNA corresponding to the longest form (v1/2) and a shorter one (v3) in all CC cell lines studied. However, RHAMM was found at the protein level on cell surface only in JEG-3 cells. This discrepancy could be explained by deficient translation of RHAMM mRNA in BeWo and JAR cells.

In addition, in accordance with other authors we did not found expression of CD44 protein nor transcript in CC cell lines which is in line with our results on human tissues. This fact can be related to the hypermethylation of the CD44 promoter in CC which led to a repression of genetic expression (Novakovic et al. 2008).

Considering the above results together with the expression of HA, RHAMM and CD44 in the human biopsies we propose that JEG-3 cell line would be an accurate model to study the role of HA and RHAMM on CC. JEG-3 showed no secretion of HA into the culture media and expressed RHAMM but no CD44 on the cell surface.

We hypothesize that HA on the surrounding ECM is able to induce migration on JEG-3 cells. It is known that different molecular weight HA have different capabilities on such process (Monslow et al. 2015). While it was reported that lower doses of HA were unable to induce invasion in JEG-3 cell (Takahashi et al. 2014) using an *in vitro* approach we demonstrated that 500 µg/ml LMW-HA (but no HMW-HA) was able to induce JEG-3 cell migration and we also showed that proliferation was not affected after HA treatment. Moreover, considering that HA is from a pharmaceutical source and endotoxin-free, effects due to contaminants (as LPS or growth factors) that could affect the obtained results are avoided (Dong et al. 2016; Huang et al. 2014).

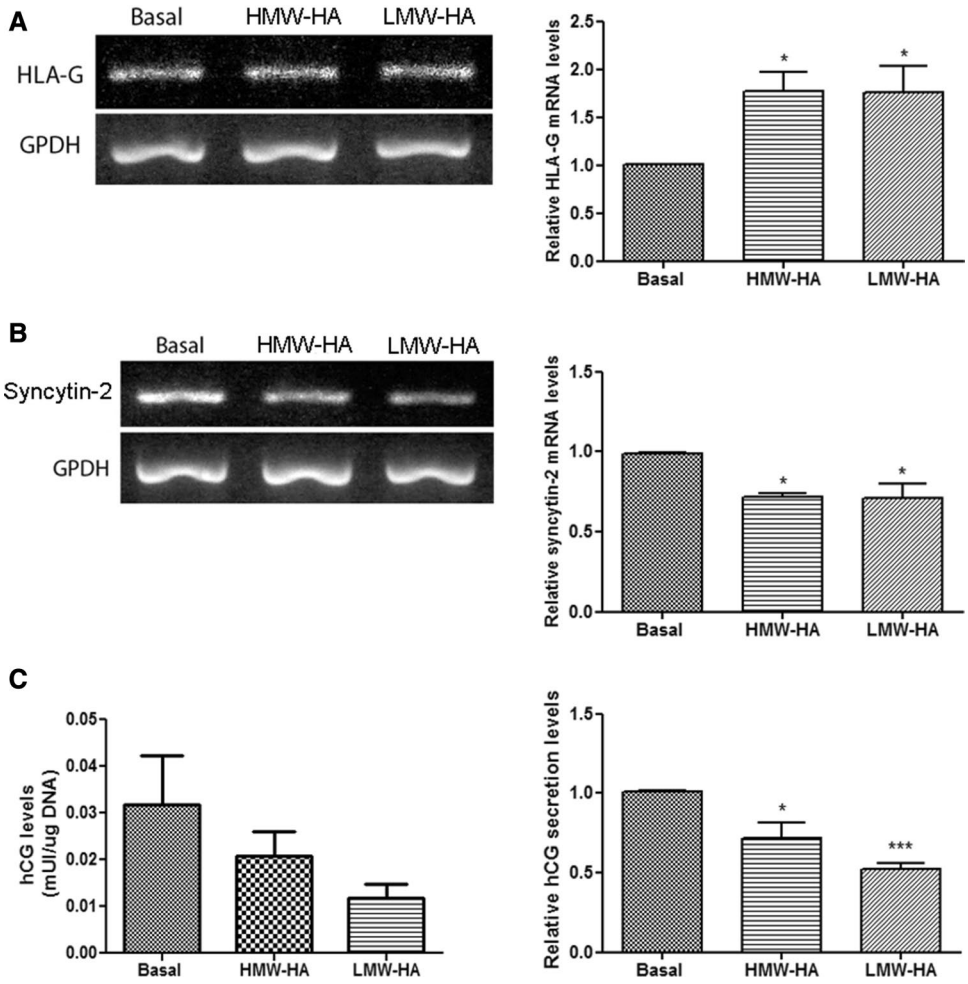

Fig. 6 Effect of HA on trophoblast markers expression. JEG-3 cells were treated with 500 µg/ml HMW-, LMW-HA or medium alone (*basal*) and then the mRNA expression of HLA-G (**A**) and Syncytin-2 (**B**) were evaluated by RT-PCR. The secretion of hCG (**C**) to the supernatant was evaluated by ECLIA. *Bars* represent the mean ± SEM of at least three independent experiments. $*P < 0.05$ and $***P < 0.001$ (respect to *basal*)

CC resembles the previllous blastocyst which is composed of three types of cells with different characteristics (Mao et al. 2007; Shih 2007). Thus, we evaluated whether HA is able to induce cell differentiation. In this work, we demonstrated that both HMW- and LMW-HA are able to increase HLA-G mRNA as well as to diminish Syncytin-2 mRNA suggesting a differentiation towards trophoblast extravillous phenotype. This population is responsible of migration and invasion into maternal tissues (Red-Horse 2004). HMW-HA contributes to tissue hydration and architecture and can be degraded to LMW-HA by Hyals and/or reactive oxygen species (ROS) in inflammatory and tumour contexts (Monslow et al. 2015). We hypothesize that fragmentation of stromal HA present in target tissues may generate fragments of LMW-HA which are able to induce CC cell differentiation and migration. It is still remain to know if LMW-HA-induced differentiation could be mediated by RHAMM.

We also hypothesize that MEK/ERK and PI3K/Akt signaling pathways, which are related to microenvironment stimulus, could mediate the HA effect previously described. Their involvement in HA effects on cancer was previously demonstrated in leukemia and fibrosarcoma by our group and others (Cordo Russo et al. 2010; Kouvidi et al. 2011; Lompardía et al. 2013; Zhu et al. 2013). We showed that after treatment with Ly249002, a decreased in cell migration was found and such effect could not be reversed by HA treatment, suggesting that HA-induced migration is PI3K-dependent. However, we were not able to show Akt modulation. Interestingly, we also found that HA treatment induced ERK phosphorylation mediated by MEK and PI3K suggesting a crosstalk between both signalling pathways. Similar situation was previously demonstrated in malignancies such as breast cancer and mesothelioma (Ebi et al. 2013; Niba et al. 2013). Furthermore, we showed for the first time that HA-induced migration is mediated by RHAMM as well as that such interaction might activate ERK. The involvement of HA-RHAMM interaction in cell migration as well as the following ERK activation was also described for others malignancies such as breast and esophageal squamous carcinoma (Hamilton et al. 2007; Twarock et al. 2010). Our results let us also to hypothesize

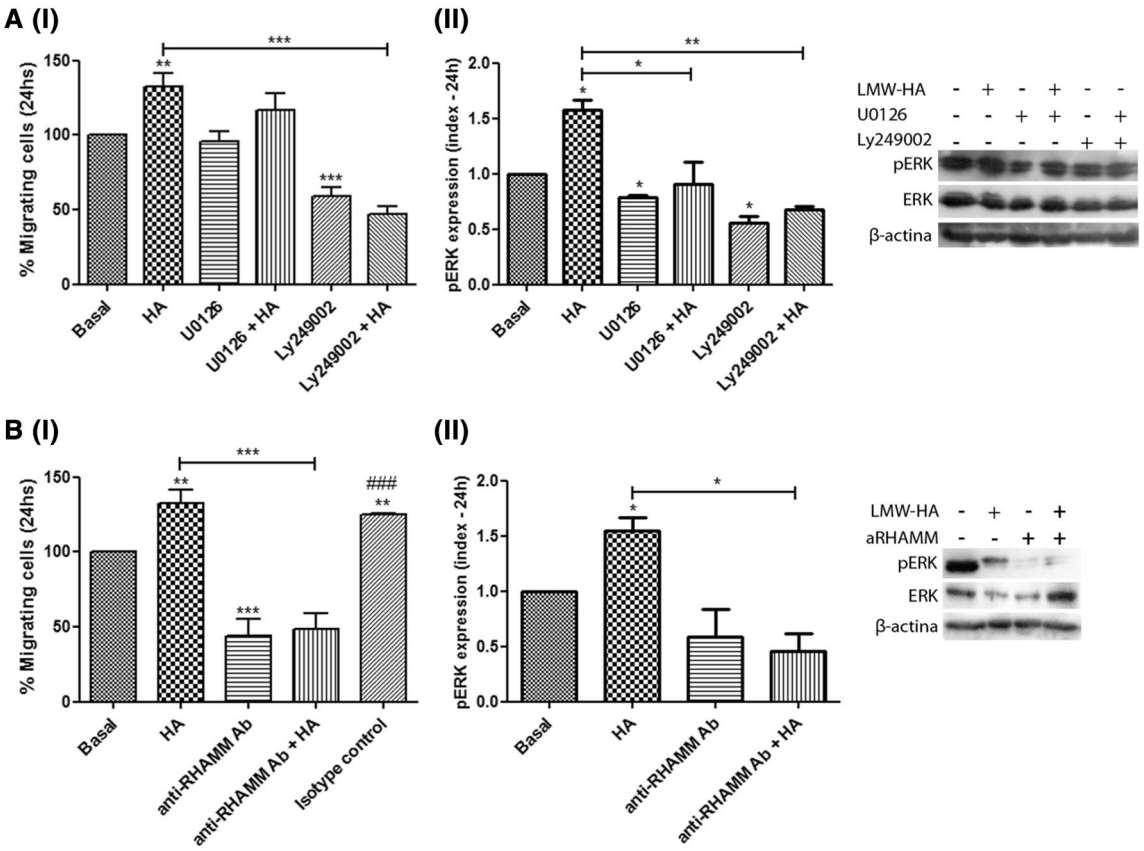

Fig. 7 Signalling pathways and receptors involved in the LMW-HA effect on migration. **A** Cells were treated with either HA, Ly294002, U0126 or combination. *(I)* Migration was evaluated by chemotaxis assay at 24h. *(II)* pERK index was evaluated by western blot at 24h to confirm whether HA activates PI3K and/or MEK signalling pathways. **B** Cells were treated with either HA, anti-RHAMM Ab or combination. *(I)* RHAMM Ab was used to determine if RHAMM was involved in the HA-induced migration. *(II)* pERK index was evaluated by western blot at 24h to determine if HA induced ERK phosphorylation mediated by RHAMM. Results are expressed as: pERK index = (pERK/βactin)/(ERK/βactin)treated/(pERK/β-actin)/(ERK/β-actin)basal. *Bars* represent the mean ± SEM of at least three independent experiments. $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ vs *basal*. $^{\#\#\#}P < 0.001$ vs anti-RHAMM Ab

that HA-RHAMM interaction could modulate migration process through PI3K activity which could act upstream to MEK kinase. Such mechanism could be inferred by the fact that inhibition of PI3K but not MEK kinase impairs migration but both kinases affect pERK/ERK levels. Even PI3K activation by HA-RHAMM interaction was also reported on vascular smooth muscle and leukemia cells (Goueffic et al. 2006; Lompardía et al. 2013). It is interesting to note that RHAMM has no transmembrane domain so it associates to other receptors such as CD44, PDGFR, EGFR, TGFbR-1, RON (Hamilton et al. 2007; Hatano et al. 2010; Park et al. 2012; Savani et al. 2001; Zhang et al. 1998). Since CD44 is not expressed by CC cells we hypothesize that RHAMM may be associated to a growth factor receptor to exert its effect.

In conclusion, we showed for the first time a differential expression of HA and RHAMM in GTD entities. We also showed that HA induce CC cell differentiation toward a migratory phenotype but only LMW-HA is able to induce migration mediated by RHAMM as well as PI3K/Akt and MAPK (Fig. 8). These findings for the first time show RHAMM protein as a novel regulatory mechanism for CC biology related to the ECM and also contribute to the understanding of GTD pathophysiology.

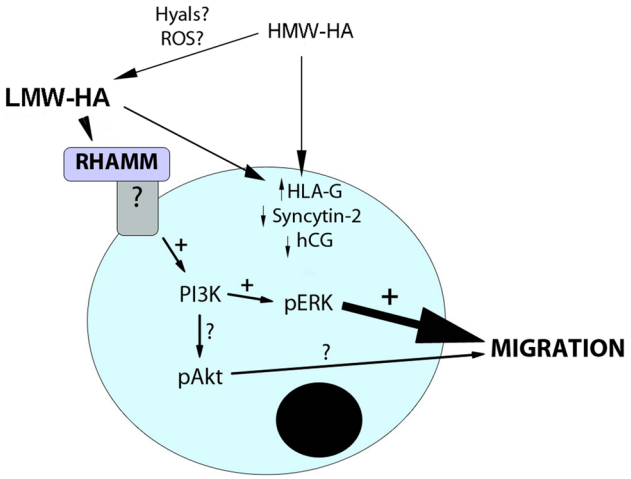

Fig. 8 Proposed model of the mechanism of action of HA on differentiation and migration of JEG-3 cells. HMW- and LMW-HA might induce extravillous trophoblast phenotype. However, LMW-HA, but no HMW-HA, might be able to induce migration through RHAMM interaction with the involvement of PI3K and MEK signaling pathways

Acknowledgements Authors wish to thank Florencia Cappuccio for help in tissue samples selection and Flavia Sacerdoti for valuable comments and suggestions on the manuscript.

Compliance with ethical standards

Funding This work was supported by grants from University of Buenos Aires (B021 and 01/W373 to SEH) and CONICET (PIP0199 to SEH).

Conflict of interest The authors declare that they have no conflict of interest.

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