

# Hepatoma-derived growth factor: from the bovine uterus to the *in vitro* embryo culture

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## Abstract

Early in cow embryo development, hepatoma-derived growth factor (HDGF) is detectable in uterine fluid. The origin of HDGF in maternal tissues is unknown, as is the effect of the induction on developing embryos. Herein, we analyze HDGF expression in day 8 endometrium exposed to embryos, as well as the effects of recombinant HDGF (rHDGF) on embryo growth. Exposure to embryos did not alter endometrial levels of *HDGF* mRNA or protein. HDGF protein localized to cell nuclei in the luminal epithelium and superficial glands and to the apical cytoplasm in deep glands. After uterine passage, levels of embryonic *HDGF* mRNA decreased and HDGF protein was detected only in the trophoctoderm. In fetal fibroblast cultures, addition of rHDGF promoted cell proliferation. In experiments with group cultures of morulae in protein-free medium containing polyvinyl alcohol, adding rHDGF inhibited blastocyst development and did not affect cell counts when the morulae were early (day 5), whereas it enhanced blastocyst development and increased cell counts when the morulae were compact (day 6). In cultures of individual day 6 morulae, adding rHDGF promoted blastocyst development and increased cell counts. Our experiments with rHDGF indicate that the growth factor stimulates embryonic development and cell proliferation. HDGF is synthesized similarly by the endometrium and embryo, and it may exert embryotropic effects by autocrine and/or paracrine mechanisms.

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## Introduction

The acidic, heparin-binding protein hepatoma-derived growth factor (HDGF) was originally isolated from a conditioned medium of human hepatoma cells (Nakamura *et al.* 1994). HDGF is a multifunctional protein that participates in the regulation of many cellular events, including ribosome biogenesis, RNA processing, DNA damage repair, and transcription (Zhao *et al.* 2011). Exogenous HDGF promotes proliferation of numerous cell types (e.g. vascular smooth muscle and endothelial and fibroblast cells; Klagsbrun *et al.* 1986, Everett *et al.* 2001), including cancer cell lines (Bernard *et al.* 2003, Hu *et al.* 2003, Chang *et al.* 2007, Lee *et al.* 2010, Liao *et al.* 2010, Yang *et al.* 2013). In tumor cells, overexpressed HDGF increases proliferation, migration, and neoangiogenesis (Sasaki *et al.* 2011, Thirant *et al.* 2012, Tsai *et al.* 2013), and it inhibits apoptosis (Lee *et al.* 2010, Liao *et al.* 2010).

HDGF is involved in organ development and tissue differentiation (Oliver & Al-Awqati 1998, Everett 2001, Everett *et al.* 2001, Enomoto *et al.* 2002). The molecular details of how HDGF influences development remain unclear because membrane or nuclear receptors for

HDGF have yet to be identified. Nevertheless, it does appear that this soluble factor promotes remodeling by activating the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway (Kung *et al.* 2012). Factors regulating *HDGF* gene expression and activity also remain unclear, though studies indicate that restoring p53 activity in cancer cells represses transcription of the *HDGF* gene (Sasaki *et al.* 2011).

In the bovine species, previous studies have already identified numerous growth factors produced by the reproductive tract, which improve *in vitro* embryo development. These include insulin-like growth factor 1 (IGF1), epidermal growth factor (EGF), activin, granulocyte-macrophage colony-stimulating factor (GM-CSF or CSF2), hyaluronan, fibroblast growth factor 2 (FGF2), and platelet-activating factor (PAF) (Gopichandran & Leese 2006, Block *et al.* 2011, Bonilla *et al.* 2011, Fields *et al.* 2011, Trigal *et al.* 2011, Sakagami *et al.* 2012). CSF2 has also been shown to act during gastrulation development (Loureiro *et al.* 2009, 2011). Several of these growth factors have been shown to improve survival-to-term following embryo transfer, including EGF, CSF2, CSF3, IGF1, and hyaluronan (Lim *et al.* 2007,

Loureiro *et al.* 2009, Block *et al.* 2011, Sakagami *et al.* 2012). In a previous study combining two-dimensional difference gel electrophoresis with liquid chromatography–electrospray ionization mass spectrometry, we identified HDGF among 23 proteins that were over-expressed in day 8 uterine fluid (UF) collected from pregnant cows (Muñoz *et al.* 2012). This suggests that this growth factor may be involved in the regulation of blastocyst development, prompting the need for i) a detailed analysis of *HDGF* gene expression and ii) first investigation of the biological actions this growth factor will exert at the endometrium–embryo interface during early pregnancy.

In this study, we examined the origin of luminal HDGF by determining the endometrial and embryonic localization of the protein and gene expression during transient *in utero* development of embryos produced *in vitro*. We also analyzed the bioactivity of recombinant HDGF (rHDGF) on fetal fibroblast cell proliferation and its effects on the *in vitro* development of bovine embryos.

## Materials and methods

All chemicals were purchased from Sigma, unless otherwise indicated.

### Animals

All experimental procedures with animals were carried out according to the European Community Directive 2010/63/EU (Spanish Regulation R.D. 53/2013) and were approved by the Animal Research Ethics Committee of SERIDA.

The experimental design has been described previously (Muñoz *et al.* 2012). Briefly, crossbred, age-matched, mixed heifers (Asturiana de los Valles and Asturiana de la Montaña cross,  $n=6$ ) and uniparous cows ( $n=5$ ) were synchronized to estrus (day 0) by using an intravaginal progesterone device (PRID ALPHA, Ceva, Barcelona, Spain) for 10 days combined with a prostaglandin analog (Dynolitic, Pfizer) injected 48 h before progestagen removal. Animals were assigned to either a cyclic group ( $n=6$ ) or an *in vitro*-produced (IVP) group ( $n=5$ ). The IVP group received 50 IVP embryos on day 5 (Gómez *et al.* 2013), while the cyclic group underwent sham transfer. Animals were killed in a nearby slaughterhouse (Matadero de Pravia, Asturias, Spain) on day 8 of their cycle.

Uterine horns were flushed, and the presence of embryos at appropriate development stages was examined in washes from embryo-transferred females. Endometrial samples and embryos were processed as described below.

### Endometrial sample collection

Genital apparatuses were transported to the laboratory at 4 °C, and middle and cranial thirds were sampled as described previously (Gómez *et al.* 2013). Briefly, endometrial cross-sections were taken from caruncular and intercaruncular regions from the horn ipsilateral to the corpus luteum. Samples were processed for immunohistochemistry (IHC), western

blotting (WB), and mRNA expression analysis. For WB and RT-PCR analysis, the caruncular and intercaruncular endometria, including deep endometrial glands, were dissected from the underlying myometrium. To allow for histological evaluation of the endometrium, IHC samples were fixed overnight at 4 °C in 4% paraformaldehyde in 0.1 M PBS (8.1 mM sodium phosphate dibasic, 137.9 mM sodium chloride, 2.07 mM potassium chloride, and 1.5 mM potassium phosphate monobasic; pH 7.4), dehydrated, embedded in paraffin, sectioned to a thickness of 4 µm, and stained with hematoxylin and eosin (Panreac, Barcelona, Spain).

Samples collected for WB were snap frozen in liquid nitrogen and stored at –145 °C until homogenization. Endometrial tissue was homogenized using a high-intensity ultrasonic processor (Sonics & Materials, Newton, MA, USA) in 10 ml lysis buffer (CellLytic, Sigma, Madrid, Spain, MT C3228) and 50 µl protease inhibitors (P8340, Sigma) per gram of tissue. After centrifugation at 3026 g for 10 min at 4 °C, the supernatant was collected and stored at –145 °C. Total protein concentration in tissue lysates was analyzed using the Quick Start Bradford protein assay kit (Bio-Rad Laboratories).

Samples for mRNA expression analysis with quantitative real-time reverse transcriptase PCR (qRT-PCR) were incubated overnight at 4 °C in RNAlater (Ambion, Huntingdon, Cambridgeshire, UK). Subsequently, excess RNAlater was removed and samples were stored at –145 °C until use.

### Analysis of HDGF protein levels in bovine endometrium by IHC

Cell-specific expression of HDGF was analyzed with a mouse polyclonal antibody against HDGF (43668, Abcam, Cambridge, UK; diluted 1:1000) in endometrial cross-sections following a classical immunostaining protocol (Muñoz *et al.* 2005). Immunostained sections were rinsed in tap water, counterstained using modified Harris hematoxylin solution (AHHSS16), cleared, and mounted. Images of representative fields were recorded under bright-field illumination using an Olympus BX51 microscope fitted with an Olympus DP70 digital camera. Positive immunostaining was scored by two independent observers blind to the nature of the tissue. Negative control sections were incubated with 5% normal blocking serum instead of the primary antibody. No significant positive staining was observed in negative control sections (data not shown).

### Analysis of HDGF protein levels in bovine endometrium by WB

Immunoblotting was performed as described previously (Muñoz *et al.* 2012). Briefly, total endometrial protein (5 µg/sample) was electrophoresed on 12% SDS–PAGE gels. Proteins were then transferred electrically onto a PVDF membrane (Millipore, Billerica, MA, USA), which was incubated with a mouse polyclonal antibody against HDGF (43668, Abcam; diluted 1:1500) and a mouse MAB against β-actin (2228, Sigma; diluted 1:6600). After washing, the membrane was incubated with the required secondary antibody (goat anti-mouse/HRP conjugate; Amersham Pharmacia Biotech) at 1:4000 dilution and detected using the Pierce ECL or ECL

plus detection kits (Thermo Scientific, Waltham, MA, USA). Protein levels were quantified using scanning densitometry and the ImageJ Software (<http://rsbweb.nih.gov/ij/>), with three replicate lanes quantified per biological condition. Differences in mean protein abundance were analyzed and expressed as fold change (least square mean (LSM)  $\pm$  S.E.M.). As levels of  $\beta$ -actin vary significantly across uterus regions and even between replicate samples, protein expression was normalized based on protein loading visualized by Coomassie staining.

### Analysis of HDGF mRNA levels in bovine endometrium by real-time RT-PCR

Total RNA from frozen endometrial tissue was extracted and purified, and its quality was assessed as described previously (Mansouri-Attia *et al.* 2009). Total purified RNA (1  $\mu$ g) was reverse transcribed in a final reaction volume of 20  $\mu$ l using Superscript III enzyme (Invitrogen) and random primers (Roche). Reaction conditions were 65 °C for 10 min followed by 5 min cooling on ice to allow annealing, 42 °C for 50 min to allow synthesis, and finally 70 °C for 15 min to denature the enzyme.

This reverse-transcribed DNA was then subjected to real-time RT-PCR using primers targeting the *HDGF* gene or one of the following reference genes:  $\beta$ -actin (*ACTB* (*ACT*)); chromosome 11 open reading frame, human *C2orf29* (*CNOT11* (*C2ORF29*)); glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*); ribosomal protein L19 (*RPL19*); solute carrier family 30 (zinc transporter), member 6 (*SLC30A6*); and succinate dehydrogenase flavoprotein subunit A (*SDHA*). Primers for all genes were designed using Primer Express version 3.0 (Applied Biosystems; Table 1). Real-time PCRs were carried out using Master Mix SYBR Green and the Step One Plus system (Applied Biosystems), in a total volume of 15  $\mu$ l containing 5  $\mu$ l cDNA (diluted 1:200) and 0.3  $\mu$ M primers. Amplification conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 45 cycles at 95 °C for 15 s (denaturation), and 60 °C for 1 min (primer annealing and extension). Melting curve analyses were performed immediately after amplification; all samples were

analyzed on the same plate to minimize variation between runs. A calibration curve was produced using cDNA retro-transcribed from total RNA pooled from all endometrial samples (several replicates) and serially diluted as described previously (Mansouri-Attia *et al.* 2009).

All samples were reverse transcribed and amplified in duplicate. Negative control samples for each gene were run in parallel without template or without primers. None of the negative control reactions showed amplicons. Amplicon sequences for all target genes were verified by DNA sequencing (Beckman Coulter Genomics, Takeley, UK).

QRT-PCR data were analyzed using the qBase plus Software (Biogazelle, Ghent, Belgium). Cq values were transformed into normalized relative quantities (NRQs) based on several reference genes and after correcting for differences in amplification efficiency, using the approach described by Hellemans *et al.* (2007). To identify the reference genes best suited for calculating the required normalization factors (NFs), we used qBase to calculate two quality measures for the six reference genes mentioned above: the coefficient of variation of the normalized reference gene expression levels and the geNorm stability *M*-value. Subsequently, the data for another, less stable reference gene were integrated to give NFs, following the procedure described by Vandesompele *et al.* (2002). Using this stepwise approach, we determined that Cq data for *SLC30A6*, *CNOT11*, and *RPL19* were best suited for normalizing the HDGF data.

### In vivo embryo sample collection for protein and gene expression analysis

Embryos were washed three times in PBS–polyvinylpyrrolidone and grouped according to their development stage (degenerated, morulae, blastocyst, expanded blastocyst, and hatched blastocyst). Only expanded blastocysts were further processed for qRT-PCR and IHC.

Embryos for IHC were fixed for 20 min at room temperature in 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Fixed embryos were stored in 0.1 M PBS (pH 7.4) at 4 °C until use.

**Table 1** Primers used to amplify regions of the *HDGF* gene in bovine endometrium and embryos or to amplify regions of reference genes in endometrium (*SLC30A6*, *CNOT11*, and *RPL19*) and embryos (*GAPDH*, *SDHA*, and *YWHAZ*).

Symbols	Names	Accession nos	Primer sequences	Concentrations ( $\mu$ M)	Fragment length (bp)
<i>HDGF</i>	Hepatoma-derived growth factor	NM_175832.2	GACCCACGAGACGGCATT GCTGAACCCCTTCTCTTGTTG	0.3 0.3	97
<i>SLC30A6</i>	Solute carrier family 30 (zinc transporter), member 6	NM_001075766.1	TGATGAGGAAACCTAGCCCTGCC TCGGGCTGCTCCAAAAGCGT	0.3 0.3	142
<i>CNOT11</i>	Chromosome 11 open reading frame, human <i>C2orf29</i>	XM_582695.5	CCTTCAAGAGCCCCTGT GGGTCTTTTCCAACCTCC	0.3 0.3	64
<i>RPL19</i>	Ribosomal protein L19	NM_001040516	CCCCAATGAGACCAATGAAATC CAGCCCATCTTTGATCAGCTT	0.3 0.3	73
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	XM_618013	TTCAACGGCACAGTCAAGG ACATACTCAGCACCAGCATCAC	0.2 0.2	119
<i>SDHA</i>	Succinate dehydrogenase flavoprotein subunit A	NM_174178	GCAGAACCTGATGCTTTGTG CGTAGGAGAGCGTGTGCTT	0.3 0.3	185
<i>YWHAZ</i>	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide	BM446307	GCATCCCACAGACTATTCC GCAAAGACAATGACAGACCA	0.2 0.2	120



Embryos for qRT-PCR were snap frozen in Eppendorf DNA LoBind tubes (catalog no. 022431021, Eppendorf, Hamburg, Germany) and stored at  $-145^{\circ}\text{C}$  until use.

### **Analysis of HDGF protein expression in bovine embryos by IHC**

HDGF protein expression was analyzed by IHC in day 8 blastocysts ( $n=8/\text{condition}$ ) using a mouse polyclonal primary antibody against HDGF (43668, Abcam) as described previously (Muñoz *et al.* 2008). Serial optical sections were acquired by confocal laser scanning microscopy in order to characterize the pattern of HDGF expression. Negative controls using only secondary antibody were performed in parallel.

### **Quantification of HDGF mRNA levels in bovine embryos by qRT-PCR**

RT-PCR was performed with embryos according to Khan *et al.* (2012), with some modifications. Briefly, RNAs from ten embryos were extracted using the Picopure RNA isolation Kit (Arcturus, Applied Biosystems) and incubated in extraction buffer containing a mixture of Luciferase RNA (Promega) and 16S–18S carrier RNA (Roche Diagnostics) at  $42^{\circ}\text{C}$  for 30 min, according to the manufacturer's instructions. Rates of mRNA recovery were evaluated by optical density measurement at a wavelength of 260 nm (using Nanodrop, Thermo Fisher Scientific, Madrid, Spain). cDNA was synthesized starting from the same number of 'equivalent embryos' in each sample and using the following conditions:  $25^{\circ}\text{C}$  for 5 min to allow annealing,  $50^{\circ}\text{C}$  for 60 min for RT, and  $70^{\circ}\text{C}$  for 15 min to denature the enzyme.

PCR amplification was performed using the same primers targeting *HDGF* as were used for RT-PCR of endometrial samples. Data were analyzed using the qBase plus Software (Biogazelle). In contrast to the reference genes amplified in endometrial samples, the following reference genes were amplified in embryo samples, based on Goossens *et al.* (2005) (Table 1): *GAPDH*, *SDHA*, and the gene encoding tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*). The qRT-PCRs were performed in a final volume of 25  $\mu\text{l}$  containing 10  $\mu\text{l}$  cDNA from 0.3 embryo equivalents. Cycling conditions were the same as for endometrial samples, except that 40 cycles of denaturation, annealing, and extension were performed. Amplified PCR fragments were verified by 1% agarose gel electrophoresis and ethidium bromide staining.

For amplification of each gene, all samples from a single experiment were analyzed on a single plate, and each PCR was performed in triplicate. Each experiment was conducted with five batches of embryos. The same calibration curve as for endometrial samples was used. Amplification efficiencies were  $>80\%$  and correlation coefficients were  $>0.98$ . Negative control amplifications lacking template were run in parallel for each gene, and no false positives were observed.

Ct values were transformed into NRQs using qBase plus (Biogazelle) as described for endometrial samples. Data were analyzed using the Wilcoxon–Mann–Whitney *U* test.

### **IVP of bovine embryos**

IVP embryos were obtained as described previously (Trigal *et al.* 2012), with minor modifications. Briefly, ovarian follicles with diameters of 3–8 mm from ovaries collected in a slaughterhouse were aspirated and cumulus–oocyte complexes recovered. After *in vitro* maturation for 24 h, oocytes were subjected to IVF (day 0) using frozen/thawed semen from a single bull obtained through a swim-up procedure. Embryos were cultured in synthetic oviduct fluid (SOF) containing amino acids, citrate, and myo-inositol. SOF droplets were layered under mineral oil and incubated at  $38.7^{\circ}\text{C}$  in an atmosphere of 5%  $\text{CO}_2$  and 5%  $\text{O}_2$  with saturated humidity. In order to produce morulae, SOF was supplemented with 6 mg/ml BSA (SOF–BSA) from days 1 to 5 or 6. Within experiments (i.e. from day 5 or 6 onwards), embryos were cultured in SOF with 0.5 mg/ml polyvinyl alcohol (PVA) (SOF–PVA), in groups or singly, as single culture allows discarding paracrine effects from neighboring embryos. Group cultures comprised 50  $\mu\text{l}$  droplets with 25–35 embryos/drop. Single cultures were performed in 12  $\mu\text{l}$  droplets (Muñoz *et al.* 2014a,b,c).

### **Differential cell counts and apoptosis in blastocysts**

Cell counts and apoptosis levels were measured in day 8 expanded blastocysts. Embryonic cells in the inner cell mass (ICM) and trophoctoderm (TE) were differentially counted using a propidium iodide/bisbenzimidazole method (Trigal *et al.* 2012) with minor modifications.

TUNEL staining was carried out as described previously (Rodríguez *et al.* 2006), and nuclei were classified as showing apoptotic or necrotic morphology using the criteria of Gjørret *et al.* (2003). The numbers of apoptotic cells and necrotic cells were expressed as percentages of total cells to give the apoptotic index (AI) and necrotic index respectively. AI was measured in the ICM and in the TE.

Tissues were stained for caspase 3 using a procedure described by Wydooghe *et al.* (2001) and modified in our laboratory (Gómez *et al.* 2013). Caspase protein was detected in the cytoplasm of target embryos, while negative controls showed no positive staining.

### **Synthesis of rHDGF**

Abyntek Laboratories (Derio, Bizkaia, Spain) produced rHDGF using the following procedure. Briefly, *HDGF* cDNA from bovine species was chemically synthesized with codon optimization for mammalian cell expression. A Flag tag sequence was inserted at the 5'-end of the coding region, followed by a Kozak sequence. The resulting *Flag-HDGF* cDNA was cloned into the pcDNA3.1 (–) expression vector (Life Technologies), which was transiently transfected into HEK293 cells. At 72 h after transfection, cells were harvested and HDGF protein was purified by affinity chromatography against the Flag tag. Protein purification was monitored by SDS–PAGE and WB against the Flag tag.

### Proliferation assay of bovine fetal fibroblasts cultured with rHDGF

Fibroblasts were obtained from a 3-month-old bovine fetus, passaged for multiplication, and stored in aliquots in a medium containing 90% DMSO as described previously (Muñoz *et al.* 2008). For rHDGF proliferation assay, thawed fibroblasts were routinely maintained in a basal medium (DMEM supplemented with 10% FCS, 1% non-essential amino acids, and 1% penicillin–streptomycin–amphotericin mixture) in a humidified incubator at 38.5 °C in an atmosphere of 5% CO<sub>2</sub>. Cells were treated with rHDGF in a medium with and without FCS at 24 h post-seeding and then cultured for 48 h in the presence of rHDGF. Real-time cell proliferation was measured using xCelligence System (Acea, Izasa, Barcelona, Spain). Serum-containing and serum-free conditions (as intended for *in vitro* embryo culture) were used to test rHDGF cell proliferation activity. A 96-well plate was filled with 100 µl basal media/well and equilibrated at room temperature for 30 min. The plate was placed into a station cradle (housed in a humidified incubator at 38.5 °C in an atmosphere of 5% CO<sub>2</sub>) to establish background reading. Cells were enumerated by Trypan blue staining and plated at a density of 1 × 10<sup>4</sup> cells/well in five replicates. Cells were allowed to settle for 30 min outside the incubator before returning the plate to the work station. Cell growth was monitored by electrical impedance measurements expressed as a normalized cell proliferation index (CPI).

### Experimental design

#### Experiment 1: levels of HDGF mRNA and protein in endometrium and embryos

This experiment aimed to explore the uterine and/or embryonic origins of HDGF. Endometrial tissue was analyzed by IHC to explore distribution and localization of HDGF protein, by WB to analyze HDGF levels and by qRT-PCR to measure HDGF mRNA levels. Analogously, three groups of seven expanded blastocysts (day 8) were analyzed by ICH and five pools of ten such blastocysts were analyzed by qRT-PCR. Blastocysts had been either entirely produced *in vitro* or transferred into cows on day 5.

#### Experiment 2: effect of rHDGF on bovine fibroblast cell proliferation assay

We analyzed rHDGF bioactivity using a real-time cell proliferation assay. Several rHDGF concentrations (0, 0.1, 1, 10, 100, and 250 ng/ml) were tested both in medium containing 10% FCS and in serum-free conditions (i.e. as intended to be used within embryo culture). Normalized CPI was calculated at 24 h (serum-containing conditions) and 48 h (serum-free conditions) as described for fetal fibroblasts.

#### Experiment 3: effect of rHDGF on *in vitro* embryo development

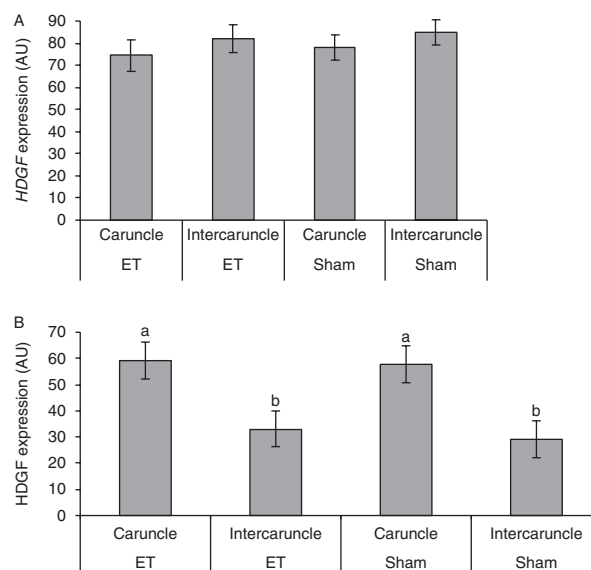
First, we analyzed group embryo cultures. On day 5, early morulae produced in SOF–BSA were selected and cultured to day 8 in SOF–PVA in the presence of rHDGF at 0, 0.1, 1, or 10 ng/ml. Embryo development was recorded and cells were differentially counted in day 8 blastocysts.

Secondly, we compared embryo development over a shorter culture period (days 6–8) in SOF–PVA in the presence of 0, 1, or 100 ng/ml rHDGF. Approximately, 95% of the embryos cultured were day 6 morulae, with the remainder early blastocysts. When the embryos had become day 8 blastocysts, their embryonic development was analyzed and differential cell counts were performed.

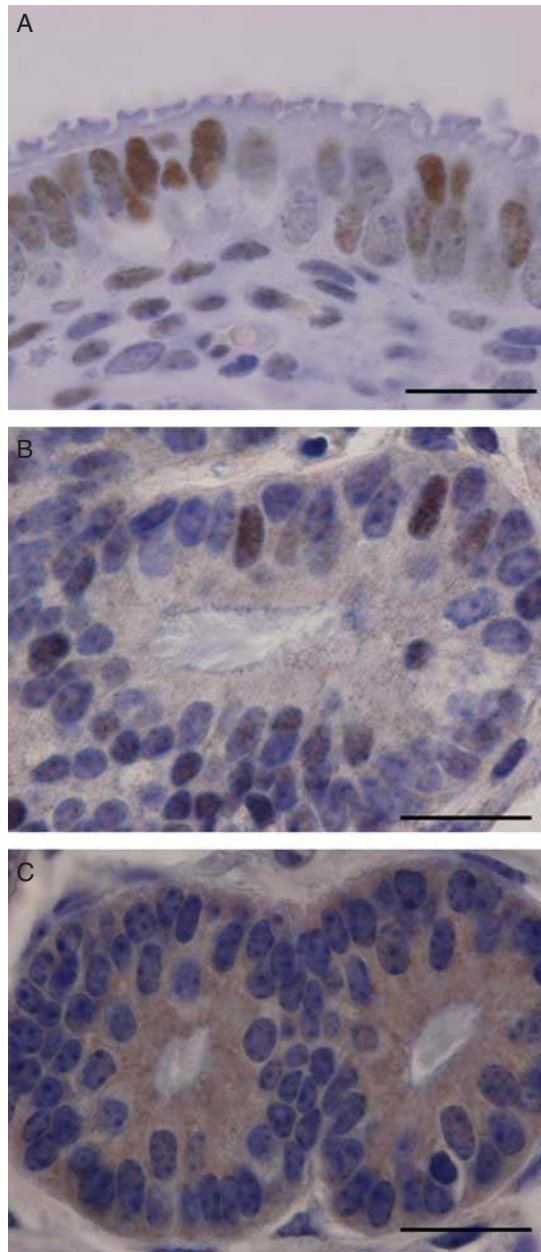
Finally, we tested the optimal culture conditions (i.e. 100 ng/ml rHDGF in SOF–PVA) in individual embryo culture from days 6 to 8. Individual culture allows discarding paracrine effects associated with embryo culture in groups. Data on embryo development were recorded daily, and day 8 blastocysts were differentially counted and subjected to TUNEL staining.

### Statistical analysis

Sources of variation affecting fibroblast proliferation (CPI), and embryo development and recovery, i.e. cell counts, apoptosis (TUNEL staining), and expression of protein and mRNA (WB and qRT-PCR), were identified by fitting linear models using the Proc GLM module of SAS/STAT (version 9.2; SAS Institute, Cary, NC, USA). The models included the following fixed effects: experimental treatment, embryonic stage, animal (where appropriate), and replicate (technical and biological). LSMs and their errors ( $\pm$  S.E.M.) were estimated for each level of fixed effects with a significant *F* value. The Ryan–Einot–Gabriel–Welsch *Q*-test was used to compare the raw means of the levels from the fixed effects.



**Figure 1** Real-time quantitative RT-PCR of HDGF mRNA (A) and western blot analysis of HDGF protein (B) from day 8 bovine endometrium collected from animals that received embryos (ET) and sham-treated control animals. Levels of mRNA and protein in caruncular and intercaruncular areas were similar between both groups of animals. HDGF protein was significantly more abundant in caruncular than in intercaruncular areas in both groups of animals. Results marked with different superscripts (a,b) are significantly different ( $P < 0.001$ ). Relative expression values are given in arbitrary units (AUs) and are plotted as the LSM  $\pm$  S.E.M.



**Figure 2** Representative immunohistochemical localization of HDGF protein in bovine endometrium. Positive labeling was found in the luminal (A) and glandular epithelial cells (B and C) and sub-epithelial stromal cells (A). In the luminal epithelium and superficial glands, immunostaining localized to the cell nuclei (A and B); in the deep glands, it localized to the apical cytoplasm adjacent to the glandular lumen (C). Scale bar, 20  $\mu$ m.

## Results

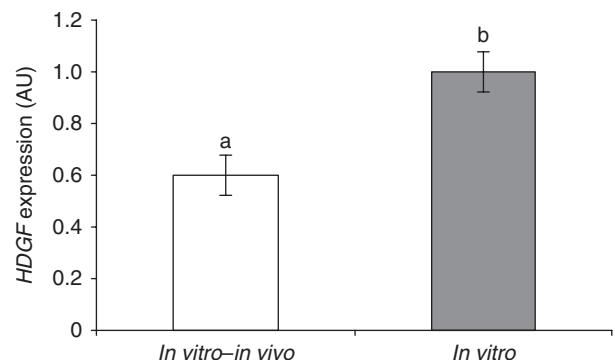
### Experiment 1: levels of HDGF mRNA and protein in endometrium and embryos

The average embryo recovery rate, expressed as the percentage of total embryos transferred, was 36.8  $\pm$  4.0% (range 10–32,  $n=5$  cows). The live embryo recovery rate was 54.1  $\pm$  4.0% (range 8–16).

Endometrial *HDGF* mRNA expression on day 8 was similar in caruncular and intercaruncular areas in the absence and presence of embryos ( $P=0.66$ ; Fig. 1A), as were mRNA levels in cranial and middle horn regions ( $P=0.70$ ; data not shown). HDGF protein levels were also similar in caruncular and intercaruncular areas in both groups of animals ( $P>0.10$ ; Fig. 1B). However, in both pregnant and cyclic animals, HDGF protein levels were significantly higher in caruncular areas (56.2  $\pm$  4.3) than in intercaruncular ones (33.3  $\pm$  4.4,  $P<0.001$ ).

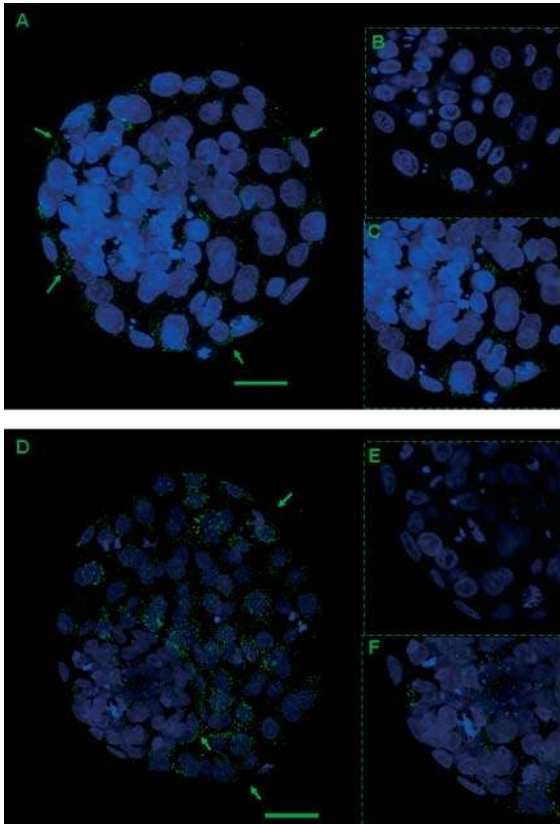
Immunoreactive HDGF was detected in all endometrial samples. HDGF protein was localized to the nuclei of the luminal epithelium, sub-epithelial stroma, and superficial gland cells (Fig. 2A and B respectively). By contrast, localization of HDGF in deep uterine glands was observed in the cytoplasm (Fig. 2C). Interestingly, some sparse cells with strongly stained cytoplasm were detected in the caruncles (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Negative control samples processed without any primary antibody showed no significant HDGF staining (Supplementary Fig. 2).

Relative abundance of *HDGF* mRNA, expressed in arbitrary units, was significantly lower in embryos recovered from the uterus (0.60  $\pm$  0.08) than in embryos produced entirely *in vitro* (1.0  $\pm$  0.08;  $P=0.016$ ; Fig. 3). HDGF protein was detected in blastocysts after uterine passage (Fig. 4A) and in blastocysts produced entirely *in vitro* (Fig. 4D). In both cases, evaluation of serial optical sections analyzed by confocal laser scanning microscopy showed positive staining in the TE (Fig. 4C and F) but none in the ICM (Fig. 4B and E). Negative control samples processed without any primary antibody showed no significant HDGF staining (Supplementary Fig. 3, see section on supplementary data given at the end of this article).



**Figure 3** Real-time quantitative RT-PCR of *HDGF* mRNA in bovine embryos. Levels of mRNA were significantly lower in blastocysts after uterine passage (*in vitro-in vivo*) than in blastocysts that developed entirely *in vitro* (*in vitro*;  $a,bP=0.016$ ).





**Figure 4** Immunofluorescence staining of HDGF protein in bovine embryos. HDGF was detected in blastocysts after uterine passage (A, B and C) and in blastocysts that developed entirely *in vitro* (D, E and F). HDGF localized to the trophoblast (TE) cells (arrows, A and D; inserts, C and F), based on staining with a mouse polyclonal antibody. Detailed inspection of single z-axis optical sections from different focal planes confirmed that HDGF was expressed exclusively in TE cells (inserts, C and F) and not in cells of the inner cell mass (inserts, B and E). Embryonic nuclei were counterstained with DAPI (blue). Scale bar, 25  $\mu$ m.

### Experiment 2: effect of rHDGF on bovine fibroblast cell proliferation assay

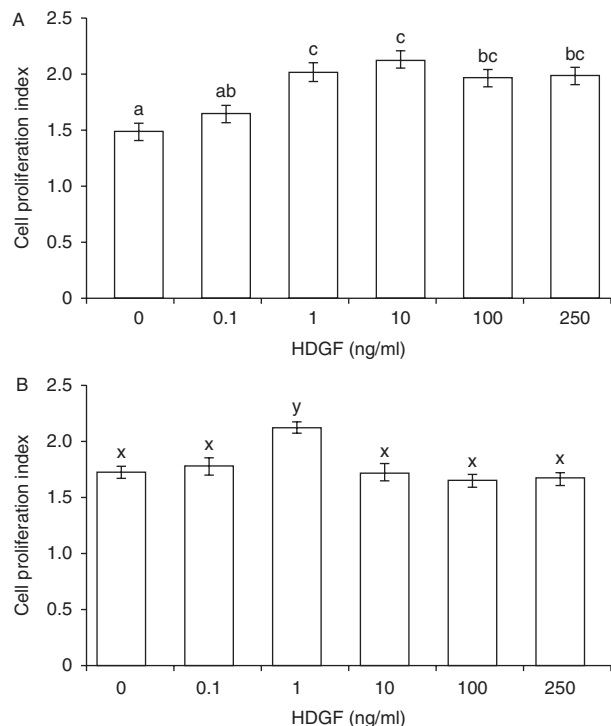
In the presence of serum, rHDGF concentrations ranging between 1 and 250  $\mu$ g/ml significantly increased the CPI ( $P < 0.02$ ; Fig. 5A). In the absence of serum, only 1  $\mu$ g/ml rHDGF showed a highly significant effect ( $P < 0.001$ ; Fig. 5B) on CPI. Representative real-time fibroblast cell growth curves are shown in Supplementary Fig. 4A, see section on supplementary data given at the end of this article (serum-containing) and Supplementary Fig. 4B (serum-free). Therefore, we initially selected 1  $\mu$ g/ml rHDGF and its closer concentrations (i.e. 0.1 and 10  $\mu$ g/ml) to analyze within *in vitro* embryo culture experiments.

### Experiment 3: effect of rHDGF on *in vitro* embryo development

We analyzed how adding rHDGF to the culture medium affects *in vitro* embryo development in SOF-PVA. First,

we analyzed group cultures. On day 5, early morulae were selected and cultured up to day 8 in the presence of rHDGF at concentrations of 0, 0.1, 1, or 10 ng/ml (Table 2). All rHDGF concentrations significantly reduced blastocyst expansion rates on day 7 ( $P < 0.01$ ), but none of the rHDGF concentrations affected blastocyst development on day 8. Nevertheless, 0, 1, and 10 ng/ml rHDGF reduced day 8 hatching rates. Addition of rHDGF at any concentration did not significantly affect cell counts in the ICM or TE on day 8 (Table 3).

To gain more insight into whether the observed effects of rHDGF depend on embryo developmental stage, we repeated the experiments with day 6 morulae cultured to day 8 using rHDGF at a concentration of 1 or 100 ng/ml in SOF-PVA. We increased the upper concentration up to levels at which other uterine growth factors (i.e. EGF and IGF1) that activate the same downstream pathways as HDGF increased pregnancy rates using embryos cultured in similar conditions (Block & Hansen 2007, Sakagami *et al.* 2012). Adding 100 ng/ml rHDGF to the culture medium significantly increased the rate of formation of day 7 morulae and day 8 blastocyst and expanded blastocysts ( $P < 0.05$ ; Table 4). Counts in TE and total cell numbers increased with 100 ng/ml rHDGF (Table 5). The adverse effects on day 7 expansion rates observed when 1 ng/ml HDGF was added to the culture medium on day 5 were not observed when an equal



**Figure 5** Cell proliferation index (CPI) of fetal fibroblasts growing in cultures with several rHDGF concentrations in the presence (A) and the absence (B) of 10% FCS. Cell growth was permanently monitored, and the CPI calculated at 48 h (A) and 24 h (B). Superscripts show significant differences: <sup>a,b,c</sup> $P < 0.02$ ; <sup>x,y</sup> $P < 0.001$ .

**Table 2** *In vitro* development of early bovine morulae cultured from days 5 to 8 in groups in synthetic oviduct fluid containing recombinant HDGF (rHDGF).

rHDGF (ng/ml)	n	Morulae (%)		Day 7 blastocysts (%)		Day 8 blastocysts (%)		
		Day 6	Day 7	Total	Expanded	Total	Expanded	Hatched
0	96	84.7±2.4	67.0±3.3	42.7±5.2	19.2±2.0*	52.1±3.8	33.2±4.9	3.3±0.7 <sup>†</sup>
0.1	96	86.8±2.4	60.7±3.3	26.0±5.2	5.8±2.0 <sup>†</sup>	49.0±3.8	25.2±4.9	0.0 <sup>§</sup>
1	97	85.6±2.5	75.2±3.3	38.0±5.2	8.1±2.0 <sup>†</sup>	56.7±3.8	35.7±4.9	0.1±0.7
10	97	88.3±2.4	75.3±3.3	41.5±5.2	9.3±2.0 <sup>†</sup>	59.1±3.8	34.5±4.9	0.0 <sup>§</sup>

n, day 5 cultured early morulae. Data were collected from four replicates. Values in the same column marked with different symbols indicate significant differences: \*<sup>†</sup>P<0.01 and \*<sup>§</sup>P<0.05.

concentration of growth factor was added on day 6. Although not tested in the same experimental replicates, cell counts in the ICM were numerically lower in expanded blastocysts cultured in groups from day 5 onwards (Table 3) than from day 6 onwards (Table 5).

Then, we analyzed the effects of rHDGF on the development of individual embryo cultures from days 6 to 8 in SOF-PVA. This technique allows for efficient, non-invasive sex detection (Muñoz *et al.* 2014a) and prediction of pregnancy viability based on spectroscopic analysis of embryo-conditioned culture medium (Muñoz *et al.* 2014b,c).

Adding rHDGF to the culture medium led to a significantly higher day 7 morulae rate ( $P<0.03$ ) as well as a higher day 7 blastocyst rate ( $P<0.01$ ) (Table 6). Blastocyst and expanded blastocyst development tended to be higher ( $P<0.07$ ) in the presence of rHDGF. Addition of rHDGF to the culture medium (Table 7) significantly increased cell counts in the ICM and total cell counts ( $P<0.03$ ), and the increases observed in the TE tended to be significant ( $P<0.06$ ). Individual embryo cultures in our experiments showed similar levels of apoptosis based on TUNEL staining (data not shown).

## Discussion

This study is the first detailed characterization of HDGF expression and function during early pregnancy in mammals. A previous work identified bovine HDGF in the UF (Muñoz *et al.* 2012), prompting us to ask whether the embryo and/or the mother express HDGF and how HDGF affects embryo development. The results of this study suggest that the presence of developing embryos does not significantly affect the levels of HDGF mRNA or protein in the endometrium. Nevertheless, HDGF mRNA and protein are detectable in embryos produced *in vitro* and in embryos exposed to the uterine environment. Exposing day 5 morulae to the uterus downregulates HDGF mRNA expression in recovered day 8 blastocysts to levels lower than those in embryos that developed entirely *in vitro*. Proliferation assay with bovine fetal fibroblasts demonstrated that rHDGF is a bioactive peptide. Experiments with embryos cultured individually or in groups in the presence of various concentrations of bioactive rHDGF indicate that this

growth factor improves embryo development and cell counts *in vitro*. Our findings suggest that HDGF may act as a paracrine and/or autocrine factor to regulate embryo development *in vivo*.

Growing evidence points to the involvement of HDGF in controlling early embryo development, raising the question as to what molecule(s) triggers production of this growth factor in the first place. One candidate is hepatocyte growth factor (HGF), an angiogenic factor (Okada *et al.* 1999) that induces HDGF expression in cancer cells (Mao *et al.* 2008, Lee *et al.* 2010). In fact, the downstream effects of HGF may be mediated in part by HDGF induction of vascular endothelial growth factor (Mao *et al.* 2008, Lee *et al.* 2010) and angiogenesis via paracrine mechanisms (Sasaki *et al.* 2011, Thirant *et al.* 2012, Tsai *et al.* 2013).

To take into account the possibility that HDGF is regulated at transcriptional and/or post-transcriptional levels, we assayed levels of both mRNA and protein in our experiments. In this study, the presence of embryos did not alter endometrial HDGF mRNA expression in caruncular and intercaruncular areas, nor did it affect the localization of HDGF protein to cranial or middle horn regions. HDGF staining patterns only differed by structure: localization was primarily cytoplasmic with apparent secretion in deep uterine glands, while the protein localized to the nucleus in the luminal epithelium and superficial glands. In addition, some sparse positive cells were found in the caruncles. The lack of regulation of endometrial HDGF expression in the uterus by the embryo is intriguing, as P4 does not control HGF expression in the ruminant uterus either (Mitko *et al.* 2008, Satterfield *et al.* 2008). However,

**Table 3** Differential cell counts in day 8 expanded blastocysts cultured in groups from days 5 to 8 in synthetic oviduct fluid containing PVA with recombinant HDGF (rHDGF).

rHDGF (ng/ml)	n	ICM	TE	Total
0	20	14.4±1.9	113.6±5.6	128.7±5.7
0.1	19	14.0±2.0	110.0±6.6	123.7±6.0
1	29	15.4±1.6	111.8±5.0	131.1±4.7
10	29	15.6±1.6	117.3±4.8	133.2±4.7

ICM, inner cell mass; n, number of embryos; TE, trophectoderm. Data were collected from four replicates. None of the values within each column indicated significant differences from one another ( $P>0.10$ ).



**Table 4** *In vitro* development of bovine morulae cultured from days 6 to 8 in synthetic oviduct fluid with PVA containing recombinant HDGF (rHDGF).

rHDGF (ng/ml)	n	Day 7 morulae (%)	Day 7 blastocysts (%)		Day 8 blastocysts (%)		
			Total	Expanded	Total	Expanded	Hatched
0	139	81.8±2.1*	42.2±4.0	13.8±2.6	54.2±3.0 <sup>‡</sup>	39.0±2.9 <sup>‡</sup>	4.7±1.5
1	99	78.9±2.4*	47.4±4.7	12.8±3.0	67.2±3.4	52.2±3.3	8.4±1.8
100	99	96.8±2.4 <sup>†</sup>	57.8±4.7	18.2±3.0	70.9±3.4 <sup>§</sup>	54.7±3.3 <sup>§</sup>	8.3±1.8

n, day 6 cultured embryos (90% morulae + early blastocysts). Data were collected from five replicates. Values in the same column with different symbols indicate significant differences: \*<sup>†</sup>P<0.01 and \*<sup>§</sup>P<0.05.

luminal and glandular epithelial cells express *HGF* receptor and epithelial c-met proto-oncogene (*MET*), under P4 regulation (Mitko *et al.* 2008, Satterfield *et al.* 2008). On the embryonic side, passage through the uterine tract led to reduced levels of *HDGF* mRNA in recovered day 8 blastocysts in comparison to those produced entirely *in vitro*. Interestingly, no differences were found in *HDGF* expression or distribution in TE cells, between both types of embryonic samples analyzed. In line with our findings, expression of *MET* has been localized to the ovine TE (Chen *et al.* 2000a,b).

We postulate that embryonal and uterine *HDGF* may act in an autocrine and/or paracrine way with the possible participation of *HGF* to induce *HDGF* synthesis and secretion in deep uterine glands and regulate *HDGF* gene expression. This crosstalk may lead the embryo to downregulate its own *HDGF* transcription to compensate for the presence of maternal *HDGF*.

In contrast to the cytoplasmic and secretory localization of *HDGF* in deep glands, we found the protein to localize to the nucleus in superficial glands and epithelium. This nuclear localization suggests that the protein was not secreted from these regions. Consistent with our findings, *HDGF* localizes to the nucleus and cytoplasm in human fibroblasts (Abouzed *et al.* 2004).

In addition to providing first evidence of endometrial and embryonic localization and gene expression of *HDGF*, our study demonstrates that the growth factor is potentially involved in regulating *in vivo* and *in vitro* development. We performed proliferation assays with rHDGF in more usual conditions with serum, whereby *HDGF* bioactivity has been previously shown with fibroblasts not only in other species (Klagsbrun *et al.* 1986, Abouzed *et al.* 2005, Wang *et al.* 2011), but also in defined conditions, as intended for embryo culture. Serum may provide carriers and cofactors necessary for *GFs* to exert their physiological effects (Francis 2010). However, a chemically defined medium is interesting for the normalization of embryo culture conditions and absence of sanitary risks associated with the compounds of animal origin. The effects of *HDGF* on *in vitro* embryo development depended on the developmental stage. When *HDGF* was added to group cultures of early morulae from day 5 onwards, blastocyst expansion on day 7 was severely reduced and blastocyst cell counts

were unchanged. By contrast, adding *HDGF* to group cultures of late morulae on day 6 promoted embryo development and increased TE cell counts. Such time-dependent effects of growth factors on *in vitro* embryo development have also been reported for *FGF2*, activin, *CSF2*, *IGF1*, *TGFβ* (*TGFB*), *TGFα* (*TGFA*), and *PDGF* (*PDGFA*; Larson *et al.* 1992a,b, Loureiro *et al.* 2009, Fields *et al.* 2011, Trigal *et al.* 2011, Dobbs *et al.* 2013). In fact, the developmental stage at which *CSF2* and *IGF1* are added to cultures strongly affects the yield and quality of the resulting blastocysts, as well as pregnancy and birth rates (Loureiro *et al.* 2009). A well-established explanation for such stage-specific effects relies on the timed expression of growth factor receptors in the embryo, which renders it responsive to uterine growth factors only during specific periods of development. There was also an apparent, side effect of the day and/or developmental stage of replacement of *BSA* by *PVA* on the *ICM* cells (day 5 vs day 6). Thus, in group culture, *ICM* cell numbers were lower with *PVA* added on day 5 than on day 6, suggesting that removal of protein from culture out of certain limits might compromise embryonic quality.

Our results suggest that *HDGF* helps to advance the day 6 morulae throughout blastulation. In bovine blastocysts, *E-cadherin* and *vimentin* are TE markers during the epithelial to mesenchymal transition (*EMT*; Yamakoshi *et al.* 2012), with each protein serving essential and specific roles at different stages of blastocyst development. Interestingly, *HDGF* downregulates *E-cadherin* expression and upregulates *vimentin* expression to help drive *EMT* in both healthy and cancer cells (Chen *et al.* 2012, Tsai *et al.* 2013). Future research should explore stage-dependent effects of

**Table 5** Differential cell counts in day 8 expanded blastocysts cultured in groups from days 6 to 8 in synthetic oviduct fluid containing *PVA* with recombinant *HDGF* (rHDGF).

rHDGF (ng/ml)	n	ICM	TE	Total
0	27	25.9±2.6	115.3±5.2 <sup>  </sup>	138.6±5.8 <sup>‡</sup>
1	37	27.4±2.2	108.3±4.4*	132.9±5.2 <sup>‡</sup>
100	38	23.2±2.7	132.2±5.4 <sup>†,¶</sup>	155.4±4.9 <sup>§</sup>

ICM, inner cell mass; n, number of embryos; TE, trophectoderm. Data were collected from four replicates. Values in the same column with different symbols indicate significant differences: \*<sup>†</sup>P<0.01 and \*<sup>§</sup>P<0.05 or a tendency, <sup>||,¶</sup>P<0.07.

**Table 6** *In vitro* development of bovine morulae cultured individually from days 6 to 8 in droplets of synthetic oviduct fluid with PVA containing 100 ng/ml recombinant HDGF (rHDGF).

rHDGF (ng/ml)	n	Day 7 (%)		Day 7 blastocysts (%)			Day 8 blastocysts (%)			
		Morulae	Total	Blastocysts	Expanded	Total	Blastocysts	Expanded	Hatched	
0	229	74.1 <sup>†</sup>	55.2	32.2 <sup>*</sup>	15.3	62.5	52.1 <sup>  </sup>	37.2 <sup>  </sup>	6.4	
100	230	91.0 <sup>§</sup>	68.4	49.5 <sup>†</sup>	20.6	76.7	67.7 <sup>¶</sup>	50.9 <sup>¶</sup>	5.7	
LSM		±4.5	±5.1	±3.8	2.8	±6.6	5.6	±4.6	±1.6	

LSMs, least square means. n, day 6 cultured early morulae. Data were collected from 11 replicates. Values in the same column with different symbols indicate significant differences: <sup>\*</sup> $P < 0.01$  and <sup>†</sup> $P < 0.05$ ; or tends, <sup>||</sup> $P < 0.07$ .

HDGF in bovine embryo cultures related to the timed expression of E-cadherin and/or vimentin.

Embryotropic effects of rHDGF were observed with both group and single cultures. Our IHC studies showing the presence of substantial HDGF in TE but no localization in the ICM suggest that HDGF directly stimulates TE proliferation, while we speculate that proliferative effects on the ICM, clearly observed in individual culture, are likely to be mediated by a second factor in a paracrine way. Our apparent finding that HDGF does not affect apoptosis is surprising, given that HDGF overexpression inhibits caspase 3 activity in cancer cells (Liao *et al.* 2010). However, consistent with our observations, in healthy mouse fibroblasts, HDGF is dispensable for apoptotic signaling (Gallitzen-doerfer *et al.* 2008). Alternatively, this unexpected result may be an artifact reflecting the fact that a certain percentage of cells showing unequivocal morphological signs of apoptosis fail to show TUNEL staining (Leidenfrost *et al.* 2011).

HDGF appears to activate the PI3K/Akt (Kung *et al.* 2012), and/or ERK (Mao *et al.* 2008, Lee *et al.* 2010) and p38 MAPK pathways (Wang *et al.* 2011). Early bovine IVP embryos show active ERK and p38 MAPK pathways (Madan *et al.* 2005), and the ERK pathway appears to be capable of activating p38 MAPK downstream kinases in the absence of p38 MAPK activity to maintain development, showing functional redundancy. Accordingly, numerous uterine signaling factors promote bovine embryo growth *in vitro* by acting through the above pathways. These factors include EGF, TGF $\beta$ , insulin, IGF1, FGF2, leukemia inhibitory factor (LIF), activin, and PDGF (Larson *et al.* 1992a,b, Loureiro *et al.* 2009, Bonilla *et al.* 2011, Fields *et al.* 2011, Trigal *et al.* 2011, Sakagami *et al.* 2012, Dobbs *et al.* 2013, Wydooghe *et al.* 2013, Cebrian-Serrano *et al.* 2014). Even small molecules in the uterus, such as retinoic acid, can also activate PI3K/Akt (Bastien *et al.* 2006) and ERK1/2 (Persaud *et al.* 2013). Interestingly, retinoic acid also improves bovine blastocyst development *in vitro* (Rodríguez *et al.* 2006, 2007a, Gómez *et al.* 2008). As shown with these factors, adding HDGF to culture medium can improve the performance of cultures of individual embryos, allowing studies not only for cow breeding *per se* but also for model studies of human embryo

culture. These factors can also be used to treat cow donors that provide few viable oocytes by aspiration and hence require individual oocyte culturing (Hansen 2010, Wydooghe *et al.* 2013).

In our *in vitro* embryo culture study, we found that rHDGF showed embryotropic effects at a concentration of 100 ng/ml, which is similar or close to the concentration dynamic range at which other growth factors increase pregnancy rates using embryos cultured in defined and undefined conditions. Examples include bovine embryo culture in the presence of BSA and recombinant bovine CSF2 (10 ng/ml; Loureiro *et al.* 2009), as well as bovine embryo culture in the presence of PVA and human IGF1 (50 ng/ml) and mouse EGF (100 ng/ml; Sakagami *et al.* 2012) or in the presence of BSA and recombinant human IGF1 (100 ng/ml; Block & Hansen 2007). These growth factors were probably selected for inclusion in *in vitro* embryo production systems based on sequence homology with the bovine species. The pregnancy rates reported for these factors suggest functional redundancy even among factors from different species. Nevertheless, we recommend the use of homospecific growth factors based on the substantial differences we obtained in development and quality of bovine embryos *in vitro* depending on whether we used human or mouse LIF (Rodríguez *et al.* 2007b).

In this study, we have shown that HDGF is synthesized by the endometrium and embryo similarly and that it may act by both autocrine and paracrine mechanisms to promote early embryo development *in vitro* and probably *in vivo*. Adding rHDGF to IVP embryos increases blastocyst development and cell proliferation. These effects depend on the developmental stage at which the growth factor is added. HDGF seems to be an effective growth factor both in serum-containing

**Table 7** Differential cell counts in day 8 expanded blastocysts cultured individually from days 6 to 8 in single drops of synthetic oviduct fluid supplemented with PVA in the presence of 100 ng/ml recombinant HDGF (rHDGF).

rHDGF (ng/ml)	n	ICM	TE	Total cells
0	23	18.0±2.3	99.3±7.1	117.0±6.9
100	19	25.1±2.4	118.0±7.4	139.7±7.8
P value		0.03	0.06	0.03

ICM, inner cell mass; n, number of blastocysts; TE, trophectoderm. Data were collected from four replicates.

medium and also in defined culture conditions, which is advantageous for media standardization and optimization and individual tracking of cultured embryos.

## Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-14-0304>.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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