

# Inducible heat shock protein A1A (HSPA1A) is markedly expressed in rat myometrium by labour and secreted via myometrial cell-derived extracellular vesicles

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**Abstract.** The myometrium goes through physiological, cellular and molecular alterations during gestation that necessitate effective cellular proteostasis. Inducible heat shock protein A1A (HSPA1A) is a member of the 70-kDa heat shock protein A (HSPA) family, which acts as a chaperone to regulate proteostasis; however, HSPA1A also participates as a cytokine in inflammatory regulation, leading to its designation as a chaperokine. This study examined the spatiotemporal expression of HSPA1A protein in the rat myometrium throughout gestation and assessed whether it is secreted as cargo of myometrial cell-derived extracellular vesicles (EVs). Immunoblot analysis demonstrated that HSPA1A expression was markedly elevated during late pregnancy and labour and increased by uterine distension. Myometrial HSPA1A expression *in situ* increased in myocytes of longitudinal and circular muscle layers from Day 19 through to postpartum, specifically in the cytoplasm and nuclei of myocytes from both muscle layers, but frequently detectable just outside myocyte membranes. Scanning electron microscopy examination of samples isolated from hTERT-HM cell-conditioned culture medium, using EV isolation spin columns, confirmed the presence of EVs. EV lysates contained HSPA8, HSPA1A and the EV markers apoptosis-linked gene 2-interacting protein X (Alix), the tetraspanin cluster of differentiation 63 (CD63), tumour susceptibility gene 101 (TSG101) and HSP90, but not the endoplasmic reticulum protein calnexin. These results indicate that HSPA1A may act as a chaperokine in the myometrium during pregnancy.

**Keywords:** HSPA1A, HSP70, chaperokine, pregnancy, myometrium, extracellular vesicles.

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## Introduction

During pregnancy, the uterine musculature, or myometrium, is altered structurally, physiologically and biochemically under the influence of endocrine, biophysical and inflammatory signals to produce a powerful contractile tissue capable of delivering a term fetus(es) for extrauterine survival (MacPhee and Miskiewicz 2017). In the rat, where this process has been characterised in detail, the myometrium undergoes growth by proliferation and hypertrophy interspersed by activation of an intrinsic apoptotic pathway in the myometrium, yet with no indication of significant apoptosis (Shynlova *et al.* 2009, 2010, 2013). The myometrial hypertrophy as well as increased production of interstitial extracellular matrix proteins, subsequent basement membrane proteins and their integrin receptors are driven, in part, by the ever-increasing stress of uterine distension induced by growing fetuses (Shynlova *et al.* 2004, 2010; Williams *et al.* 2005, 2010). During labour, contraction-associated proteins such as gap

junction alpha-1 (GJA1) are expressed and physiological uterine inflammation occurs to enable myometrial activation and parturition (Tabb *et al.* 1992; Shynlova *et al.* 2020). Finally, in the postpartum period the uterus undergoes an involution process similar to wound healing that is marked by reorganisation of the myometrium and even cell death (Shynlova *et al.* 2007, 2008). Such a breadth of processes occurring in the myometrium during gestation necessitates the tissue to use efficient and effective cellular proteostasis.

The 70-kDa heat shock protein (HSP) family (HSPA; also known as HSP70) comprises 13 proteins encoded by 17 genes and 30 pseudogenes (Brocchieri *et al.* 2008; Kampinga *et al.* 2009). These chaperones work with other HSPs, such as the HSP90 family, and a large group of cochaperones including HSP40 (DNAJB1), stress-induced phosphoprotein-1 (STIP1; also known as Hsp70–Hsp90 organising protein (HOP)) and suppressor of tumorigenicity 13 (ST13; also known as HSP70-interacting

protein (HIP); Radons 2016). HSPA and HSP90 proteins, along with their host of cochaperones, can comprise 3% of total protein mass in unstressed cells (Finka *et al.* 2015a). Canonically, HSPA and HSP90 family members are ATP-hydrolysing chaperones that, in partnership with their cochaperones, regulate cellular proteostasis (Finka *et al.* 2015b). In this capacity, they can regulate processes such as the stress-induced unfolded protein response, the breakdown of misfolded or aggregated proteins, and modulate the complexing of macromolecules as well as protein–protein interactions, including steroid hormone receptor maturation (Echeverria and Picard 2010; Yamamoto *et al.* 2014; Sisti *et al.* 2015; Lackie *et al.* 2017). HSPA1A is the major cytosolic stress-inducible HSPA member, whereas HSPA8 (heat shock cognate 70-kDa protein (HSC70)) is the constitutively expressed essential housekeeping protein of the family (Hartl *et al.* 2011; Radons 2016).

Beyond an intracellular role, reports have now clearly demonstrated that HSPs can also be secreted and have extracellular functions (De Maio 2011). A primary mode of secretion of HSPs is via extracellular vesicles (EVs), which can be released by many different cell types in response to stress (De Maio and Vasquez 2013). EVs are comprised of lipid bilayers containing transmembrane proteins and an inner cytosol of proteins and RNA. There is a considerable EV heterogeneity in terms of biogenesis, size, cargo and function (Kalluri and LeBleu 2020). EVs can be produced by cells from cell membrane budding (microvesicles) or from multivesicular bodies of endosomal origin that release the EVs (exosomes) and their contents upon fusion with the plasma membrane (Colombo *et al.* 2014; Tkach and Théry 2016). Small EVs <200 nm in diameter are represented by exosomes as well as small microvesicles, and these small EVs are marked by the presence of proteins, including apoptosis-linked gene 2-interacting protein X (Alix), the tetraspanin cluster of differentiation 63 (CD63) and tumour susceptibility gene 101 (TSG101) (Deng and Miller 2019; Stahl and Raposo 2019; Kalluri and LeBleu 2020). HSPA1A has also been identified as cargo in EVs and implicated in promoting inflammation (De Maio 2011). Recently, minimal information required for publishing functional studies of EVs have been recommended (Théry *et al.* 2018).

There is a growing body of knowledge regarding the role of stress proteins in pregnancy and labour (MacPhee and Miskiewicz 2017). Recently, it was reported that total HSP90, including HSP90AA1 and HSP90AB1 isoforms, was detected in the myometrium throughout pregnancy (Bhatti *et al.* 2019); however, because HSPA1A has roles in proteostasis and inflammatory events, it is imperative to investigate the expression and role of HSPA1A in the myometrium. Therefore, the objectives of the present study were to examine the spatiotemporal expression of HSPA1A in the rat myometrium throughout gestation and to assess whether HSPA1A could be cargo in myometrial cell-derived EVs.

## Materials and methods

### Animals

Sprague-Dawley rats were individually housed by the University Animal Care Unit under standard environmental conditions

(12-h light–dark cycle). Rats had access to water *ad libitum* and were maintained on LabDiet Prolab (RMH 3000; PMI Nutrition International). For all experiments, virgin female rats were mated with stud males and the observation of a vaginal plug the morning after mating was designated Day 1 of the gestation period. Under these standard conditions, the time of delivery was Day 23. All experiments adhered to institutional and national standards for the care and welfare of animals and were granted ethics approval by the University Animal Care Committee under Protocols 08-02-DM to 11-02-DM.

### Tissue collection

Individual female rats were euthanised using carbon dioxide gas asphyxiation. Tissue samples were collected from animals at 10 time points: non-pregnant (NP), throughout gestation (Days 6, 12, 15, 17, 19, 21, 22 and 23 (labour)) and 1 day postpartum (PP). Four independent sets of rat uterine tissue samples were used for experiments ( $n = 4$  per time point). The labour samples collected on Day 23 were taken during active labour after the dam had delivered two to three pups.

For immunoblot analysis, the uterine horns were dissected and opened longitudinally in cold phosphate-buffered saline (PBS) solution (pH 7.4). Myometrial tissue was then isolated as described previously (Nicoletti *et al.* 2016). All samples collected were flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . For immunofluorescence detection, a portion of the intact rat uterine horn was fixed in 4% paraformaldehyde in PBS overnight at room temperature. Tissues were processed, paraffin embedded, sectioned and mounted on microscope slides as described previously (Bhatti *et al.* 2019).

To assess the effect of uterine distension on HSPA1A expression, a unilaterally pregnant rat model was used. This model has been used previously to study the effect of uterine distension or stretch on numerous genes and proteins, including the gap junction protein GJA1, the heat shock protein  $\alpha\text{B}$ -crystallin (CRYAB) and signalling kinases such as mitogen-activated protein kinase (MAPK) 14 (Ou *et al.* 1997; Oldenhof *et al.* 2002; Nicoletti *et al.* 2016). Virgin female rats were anaesthetised and underwent a unilateral tubal ligation procedure as described previously in detail by White and MacPhee (2011). Animals were monitored postoperatively and allowed to recover for at least 5 days before matings were attempted. This model results in pregnant rats each having a gravid (distended) and non-gravid (empty, non-distended control) uterine horn for analyses. Samples of gravid and non-gravid horns were collected during gestation on Days 19 and 23 ( $n = 4$  for each time point), and tissues were processed as described above.

### Immunoblot analysis

Tissue samples were homogenised using a Precellys bead mill in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) containing protease and phosphatase inhibitors, as described previously (Nicoletti *et al.* 2016). Sample protein concentrations were then determined using the Bio-Rad Protein Assay Kit II (500-0002). Protein samples (20  $\mu\text{g}$  per lane) were mixed with  $2\times$  gel loading dye (100 mM Tris-HCl, pH 6.8, 10%

**Table 1. Antibodies used for immunoblot (IB) and immunofluorescence (IF) assays**  
FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase

Antibody	Co.	Catalogue no.	Dilution
Mouse anti-Alix	Cell Signaling Technology	2171	IB: 1:5000
Rabbit anti-Calnexin	Cell Signaling Technology	2679	IB: 1:5000
Mouse anti-CD63	ThermoFisher	MA5-11501	IB: 1:10 000
Rabbit anti-DNAJB1	Enzo Life Sciences	ADI-SPA-400	IB: 1:20 000
Rabbit anti-GAPDH	Abcam	ab9485	IB: 1:10 000
Rabbit anti-HSPA1A	Abcam	ab181606	IB: 1:20 000, IF: 1:150
Rabbit anti-HSPA8	Enzo Life Sciences	ADI-SPA-816	IB: 1:5000
Mouse anti-HSP90	StressMarq	SMC-149	IB: 1:10 000
Rabbit anti-ST13	Sigma-Aldrich	HPA047116	IB: 1:2500
Mouse anti-STIP-1	Enzo Life Sciences	ADI-SRA-1500	IB: 1:10 000
Rabbit anti-TSG101	Sigma-Aldrich	HPA006161	IB: 1:5000
Goat anti-Rabbit HRP	Promega	W4011	IB: 1:10 000
Goat anti-Mouse HRP	Promega	W4021	IB: 1:10 000
Sheep anti-Rabbit FITC	Sigma-Aldrich	F7512	IF: 1:250
ChromePure Rabbit IgG	Jackson ImmunoResearch	011-000-003	IF: 1:100

$\beta$ -mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and electrophoretically separated on 10% SDS–polyacrylamide gels, followed by electroblotting to 0.2  $\mu$ m nitrocellulose membranes (162-0097; Bio-Rad). Membranes were then stained with a Reversible Protein Stain kit (PI24580; ThermoFisher Scientific) according to the manufacturer's instructions to verify protein transfer.

Nitrocellulose membranes were rinsed in Tris-buffered saline containing Tween 20 (TBST; 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) and then incubated in a blocking solution composed of 5% non-fat dry milk (w/v) in TBST. All antisera used for analyses (Table 1) were diluted in blocking solution. Blots were incubated with primary antisera overnight at 4°C with constant shaking, rinsed with TBST and probed with an appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Protein detection was accomplished using a SuperSignal West Pico chemiluminescence substrate detection system (PI34080; ThermoFisher) and multiple exposures were acquired using a Bio-Rad ChemiDoc MP digital imaging system. All membranes were subsequently stripped with Restore Western Blot Stripping Buffer (PI21059; ThermoFisher) according to the manufacturer's instructions and re-probed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein expression, which served as a loading control, using a rabbit polyclonal GAPDH-specific antibody (Table 1).

#### Immunofluorescence analysis

Tissue sections were deparaffinised, rehydrated and underwent epitope retrieval as described previously (Nicoletti *et al.* 2016). Sections were incubated for 1 h in a blocking solution consisting of 5% normal goat serum, 1% normal horse serum and 1% fetal bovine serum (FBS) in PBS. Sections were then incubated overnight at 4°C with primary antiserum or non-specific rabbit IgG (Table 1) used at the same concentration as the primary antiserum to serve as a negative control. Sections were washed with PBS, then incubated with appropriate secondary antiserum

(Table 1). Following washes in ice-cold PBS containing 0.02% Tween 20, sections were mounted in ProLong Diamond Anti-Fade Reagent with 4',6'-diamidino-2-phenylindole (P36931; ThermoFisher). An Olympus IX83 microscope equipped with an Andor Zyla 4.2 sCMOS camera (2048-pixel  $\times$  2048-pixel array; Andor USA) and CellSens software (Olympus) were used for widefield epifluorescence image capture.

#### EV isolation and biochemical evaluation

The hTERT-HM human myometrium-derived cell line was established via stable transfection of human myometrial cells with expression vectors containing the human telomerase reverse transcriptase (hTERT), which maintains telomere length and immortalises cells (Condon *et al.* 2002). These cells retain myometrial cell characteristics, such as expression of calponin (CNN1) and oxytocin receptor (OXTR) proteins (Condon *et al.* 2002). hTERT-HM cells were regularly cultivated in 75-cm<sup>2</sup> culture flasks at 37°C under 5% CO<sub>2</sub> in air using DMEM/F12 medium with L-glutamine and 15 mM HEPES (11330-032; ThermoFisher) plus 10% FBS (12483-020; ThermoFisher) and 1% penicillin–streptomycin (P/S; 15140-122; ThermoFisher). The medium was refreshed every 24 h and cells were passaged when they reached approximately 90% confluence using trypsin (0.05% (v/v) trypsin–EDTA; 15400-054; ThermoFisher). Twenty-four hours before EV collection, cells were washed three times in Opti-MEM serum-free medium (31985-070; ThermoFisher) containing 1% P/S to remove residual FBS and then cultured in 8 mL fresh Opti-MEM/1% P/S per flask. The cell culture supernatant from four flasks was pooled and centrifuged at 500g for 5 min at room temperature to remove any residual detached cells. Subsequently, the supernatant was passed through a Millex-AA 0.8- $\mu$ m syringe filter (SLAA033SS; Sigma-Aldrich) into two 50-mL tubes to remove any remaining cellular debris. The EVs were then isolated using an ExoEasy Maxi EV isolation kit (76064; Qiagen) according to the manufacturer's instructions. Briefly, the supernatant was mixed by inversion with an equal volume of Buffer XBP and

loaded onto ExoEasy affinity spin columns followed by centrifugation at 500g for 1 min. The spin columns were rinsed twice with 10 mL of Buffer XWP using centrifugation at 3000g for 5 min. The spin columns were then transferred to fresh collection tubes and EVs were eluted with 400  $\mu$ L Buffer XE. The EV eluate was concentrated to 100  $\mu$ L using a Vivaspin 500 centrifugal concentrator with a 100-kDa molecular mass cut-off (14-558-404; ThermoFisher) and then sonicated twice for 10 s each time on ice. Protein assays were conducted as described above. A 5- $\mu$ g sample of the EV preparation per lane was used for SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analyses to assess the detection of Alix, CD63, HSPA8, HSPA1A, HSP90 and TSG101 and to confirm the absence of the endoplasmic reticulum protein calnexin (Asea *et al.* 2008) using specific antisera (Table 1).

#### Scanning electron microscopy examination of EVs

For examination of EVs by scanning electron microscopy, samples were fixed for 15 min with 2.5% glutaraldehyde in Buffer XE while on 0.1% poly-L-lysine-coated glass coverslips. Samples were then washed with 0.1 M sodium cacodylate buffer (pH 7.2), followed by dehydration with an ethanol series (30%, 50%, 70%, 80%, 90%, 95% and 3  $\times$  100% ethanol; 5 min at each concentration). Following critical point drying, samples were coated with 5 nm chromium and then imaged with a Hitachi SU8010 electron microscope.

#### Data analysis

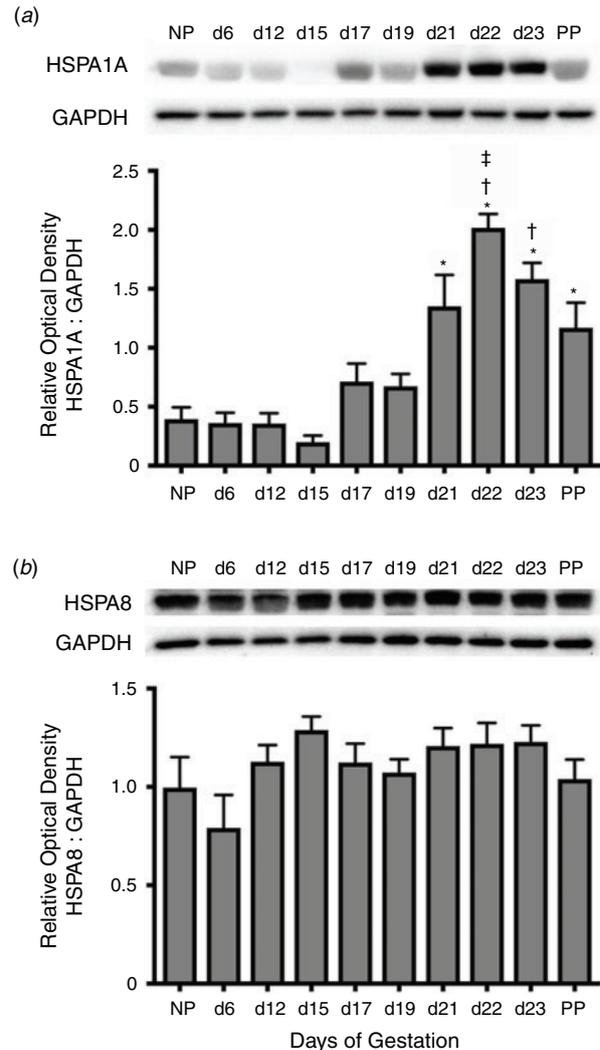
Densitometric analysis of immunoblot data was conducted using Image Lab software (Bio-Rad) and densitometric values for protein expression were normalised to the GAPDH loading control. Statistical analysis and data plotting were performed using GraphPad Prism version 8.0 for Mac (GraphPad Software; www.graphpad.com). Datasets were subjected to one-way analysis of variance (ANOVA) and post hoc Tukey–Kramer multiple comparisons tests. Statistical differences were considered significant at  $P < 0.05$ .

## Results

#### Myometrial HSPA1A expression during pregnancy

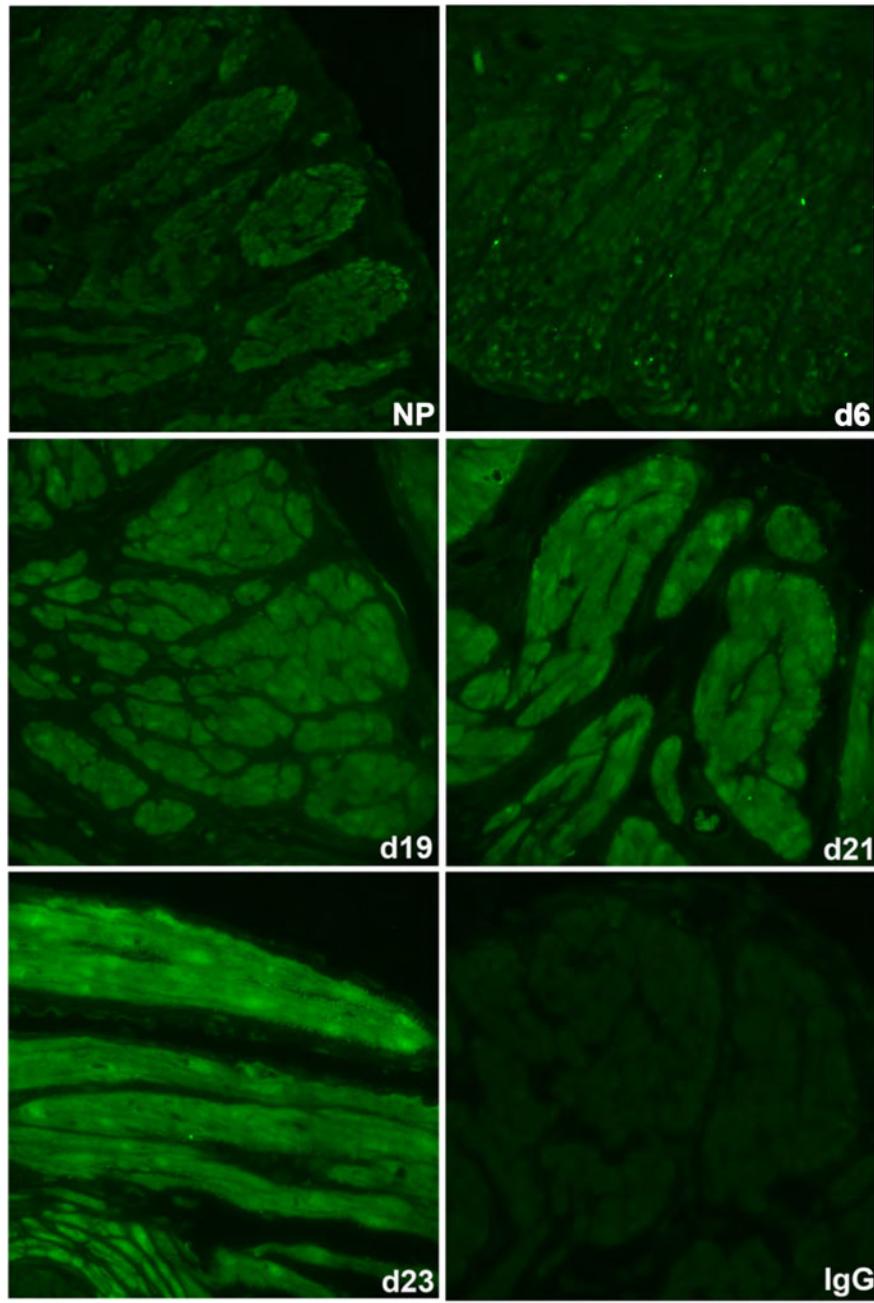
Immunoblot analysis was used to determine the temporal expression of HSPA1A during gestation (Fig. 1a). Total HSPA1A protein rapidly increased towards the end of pregnancy through to parturition. Expression of HSPA1A increased significantly from Day 21 of gestation to PP compared with NP, and Days 6, 12 and 15 of gestation ( $P < 0.05$ ), and was significantly elevated on Days 22 and 23 of gestation compared with Days 17 and 19 ( $\dagger P < 0.05$ ). HSPA1A expression on Day 22 was markedly elevated compared with PP ( $P < 0.05$ ). In contrast, levels of the essential house-keeping protein HSPA8 were not significantly altered in the myometrium during gestation (Fig. 1b).

The spatiotemporal expression of HSPA1A was examined by immunofluorescence analysis. HSPA1A was nearly undetectable in myometrium from NP to Day 6 of gestation, but expression gradually increased in myocytes of longitudinal and circular muscle layers from Day 19 of gestation through



**Fig. 1.** Analysis of inducible heat shock protein A1A (HSPA1A) and heat shock cognate 70-kDa protein (HSPA8) expression in the rat myometrium during pregnancy, parturition and postpartum. Representative immunoblots of HSPA1A, HSPA8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression are shown. (a) HSPA1A protein expression rapidly increased towards the end of pregnancy through to parturition. Expression of HSPA1A was significantly increased from Day (d) 21 of gestation to 1 day postpartum (PP) compared with non-pregnancy (NP) and Days 6, 12 and 15 ( $*P < 0.05$ ), and was significantly elevated on Days 22 and 23 compared with Days 17 and 19 ( $\dagger P < 0.05$ ). Expression on Day 22 of gestation was markedly elevated compared with PP ( $\ddagger P < 0.05$ ). (b) HSPA8 protein expression was not significantly altered in the myometrium during gestation. Data are the mean  $\pm$  s.e.m. of four independent experiments.

to PP (Figs 2, 3). HSPA1A expression appeared greater in the longitudinal than the circular layer. During this period, HSPA1A was found throughout the cytoplasm and in the nuclei of myocytes from both muscle layers (Fig. S1) and was frequently detectable in apparent extracellular areas just outside myocyte plasma membranes (Fig. 4).

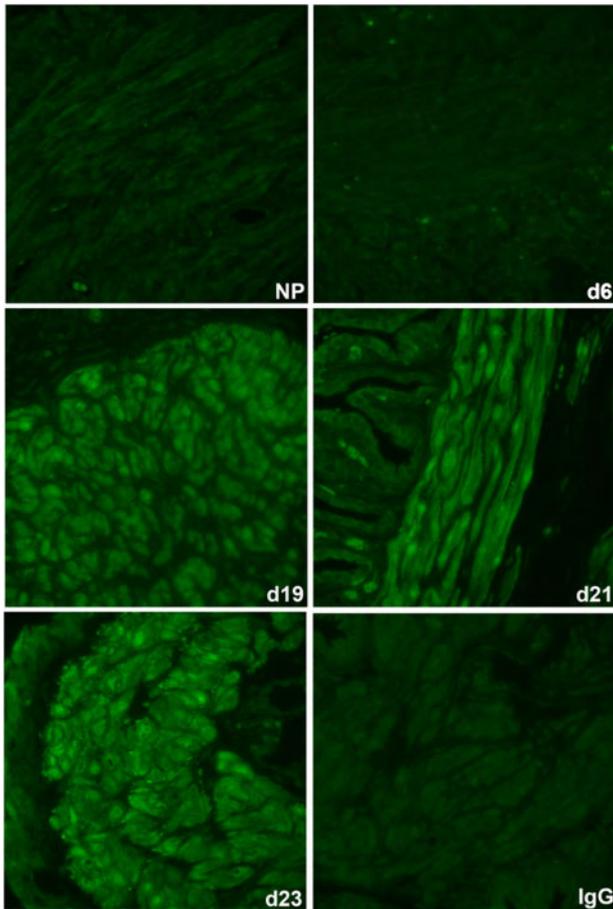


**Fig. 2.** Immunofluorescence detection of inducible heat shock protein A1A (HSPA1A) in the longitudinal muscle layer of the rat myometrium during pregnancy and parturition. Representative images from non-pregnancy (NP) and Days (d) 6, 19, 21 and 23 of gestation are shown. HSPA1A was localised to the myocyte cytoplasm as well as the nuclei, and detection increased during mid to late pregnancy and labour. IgG, non-specific rabbit IgG. Scale bar = 50  $\mu$ m.

#### *Effect of uterine distension on HSPA1A expression*

Since uterine distension resulting from fetal growth is a significant source of mechanical stress during late pregnancy, myometrial protein extracts and uterine tissue were collected from unilaterally pregnant rats at Days 19 and 23 of gestation for

both immunoblot and immunofluorescence analysis. Immunoblot analyses demonstrated that HSPA1A protein levels at Day 19 were not significantly altered by uterine distension in gravid compared with non-gravid horns (data not shown). In contrast, HSPA1A expression was significantly increased in gravid horns



**Fig. 3.** Immunofluorescence detection of inducible heat shock protein A1A (HSPA1A) in the circular muscle layer of the rat myometrium during pregnancy and parturition. Representative images from non-pregnancy (NP) and Days (d) 6, 19, 21 and 23 of gestation are shown. HSPA1A was localised to the myocyte cytoplasm as well as the nuclei, and detection increased during mid to late pregnancy and labour. IgG, non-specific rabbit IgG. Scale bar = 50  $\mu$ m.

on Day 23 compared with non-gravid contralateral horns ( $P < 0.05$ ; Fig. 5a). Similarly, immunofluorescence analysis revealed that HSPA1A detection at Day 23 was increased in the myometrium from gravid compared with corresponding non-gravid horns (Fig. 5b).

#### *Expression of HSPA1A-associated chaperones and cochaperones*

ST13 was dynamically expressed in the myometrium during pregnancy, with high levels in the early proliferative and late contractile as well as labour phases of myometrial programming (Fig. 6a). Specifically, expression on Day 6 of gestation remained similar to NP expression and levels at both time points, as well as on Days 22, 23 and PP, were significantly higher than on Days 12, 15, 17 and 19 of gestation ( $P < 0.05$ ). In contrast to ST13, STIP-1 was constitutively expressed at high levels in the myometrium throughout pregnancy (Fig. 6b), whereas DNAJB1

expression was slightly higher at Day 12 of gestation compared with Days 19, 21, 22, 23 and PP ( $P < 0.05$ ; Fig. 6c).

#### *Assessment of HSPA1A as cargo of myometrial cell-derived EVs*

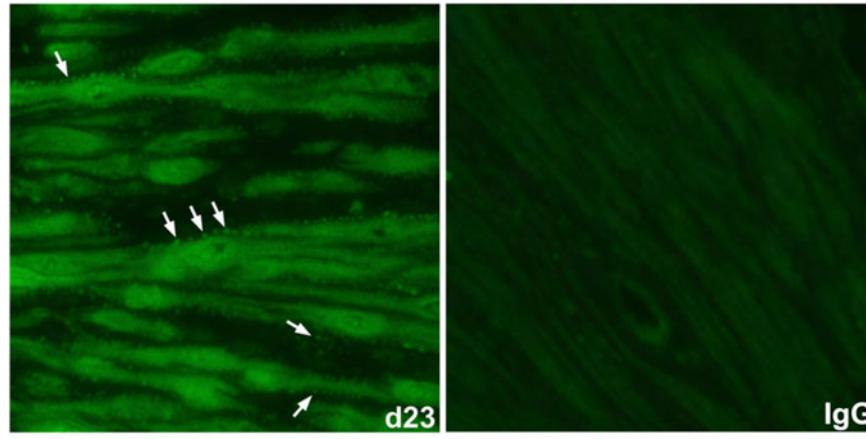
Scanning electron microscopy examination of samples isolated from hTERT-HM cell-conditioned culture medium confirmed the presence of EVs ranging from 20 to 200 nm in diameter, representative of exosomes and small microvesicles (Fig. 7a). Samples were also examined for the presence of key marker proteins in EV preparations by SDS-PAGE and immunoblot analysis. EV preparations demonstrated an enrichment of mid to high molecular weight proteins (Fig. 7b). Furthermore, EV lysates contained Alix, CD63, TSG101, HSP90, HSPA8 and HSPA1A, but not the endoplasmic reticular protein calnexin (Fig. 7c).

#### **Discussion**

HSPA1A, the major stress-inducible member of the HSPA family, is an ATP-dependent protein with important intracellular and extracellular roles, such as regulating the breakdown of misfolded or aggregated proteins and inducing inflammation respectively (De Maio and Vasquez 2013; Finka *et al.* 2015b). Nevertheless, the expression of this stress protein in the myometrium is poorly characterised. Thus, the objectives of the present study were to examine where and when HSPA1A was expressed in the rat myometrium during pregnancy and to assess whether HSPA1A could be secreted in myometrial cell-derived EVs.

#### *Intracellular HSPA1A expression*

In contrast with constitutive HSPA8 protein expression, HSPA1A protein expression in the myometrium rapidly increased towards the end of pregnancy through to parturition. Immunofluorescence detection of HSPA1A during this period was prominent throughout the cytoplasm and in the nuclei of myocytes from both longitudinal and circular muscle layers. We recently reported that the stress-induced HSP90AA1 and constitutively expressed HSP90AB1, as well as associated cochaperones p23/prostaglandin E synthase 3 (PTGES3) and activator of heat shock 90 kDa protein ATPase homologue 1 (AHSA1), were readily expressed in rat myometrium during pregnancy (Bhatti *et al.* 2019). The HSPA and HSP90 family members and their cochaperones DNAJB1, STIP1, ST13, PTGES3 and AHSA1 are important regulators of steroid hormone receptor family maturation and activation (Echeverria and Picard 2010). ST13 can also exhibit chaperone activity, aid in the interaction of HSPA1A with target proteins (Radons 2016) and was highly expressed at late pregnancy and labour in the present study, in parallel with HSPA1A expression. Using quantitative proteomics, Dhamad *et al.* (2016) showed that HSPA1A and HSPA8 were the two most abundant HSPs binding to the oestrogen receptor ER $\alpha$ , followed by HSP90AA1 and HSP90AB1. ER $\alpha$  mRNA and protein levels in the rat myometrium increase significantly during late pregnancy and labour, whereas progesterone receptor levels are readily detectable but unchanged during this period (Fang *et al.* 1996; Murata *et al.* 2003). Furthermore, HSPA1A can interact with ER $\alpha$  and associate with



**Fig. 4.** Immunofluorescence detection of extracellular inducible heat shock protein A1A (HSPA1A) in the rat myometrium during pregnancy. Representative image of the circular muscle layer at Day 23 of gestation is shown with extracellular HSPA1A detected just outside myocyte membranes (arrows). Extracellular HSPA1A was detected in both the longitudinal and circular layers of the rat myometrium from Day 19 of gestation through to Day 23 (labour). IgG, non-specific rabbit IgG. Scale bar = 25  $\mu$ m.

transcriptionally active chromatin (Dhamad *et al.* 2016). Thus, it is possible that through a HSP90–HSPA1A chaperone machine, increased myometrial HSPA1A expression during late pregnancy and labour may produce a shift to transcriptionally active, ligand-bound ER $\alpha$ –HSPA1A complexes, because HSPA1A was detected in both the cytoplasm and nuclei of myometrial cells during this period. This could facilitate ligand–ER $\alpha$  signalling as circulating concentrations of 17 $\beta$ -oestradiol rise for the initiation of parturition (Lye *et al.* 1993).

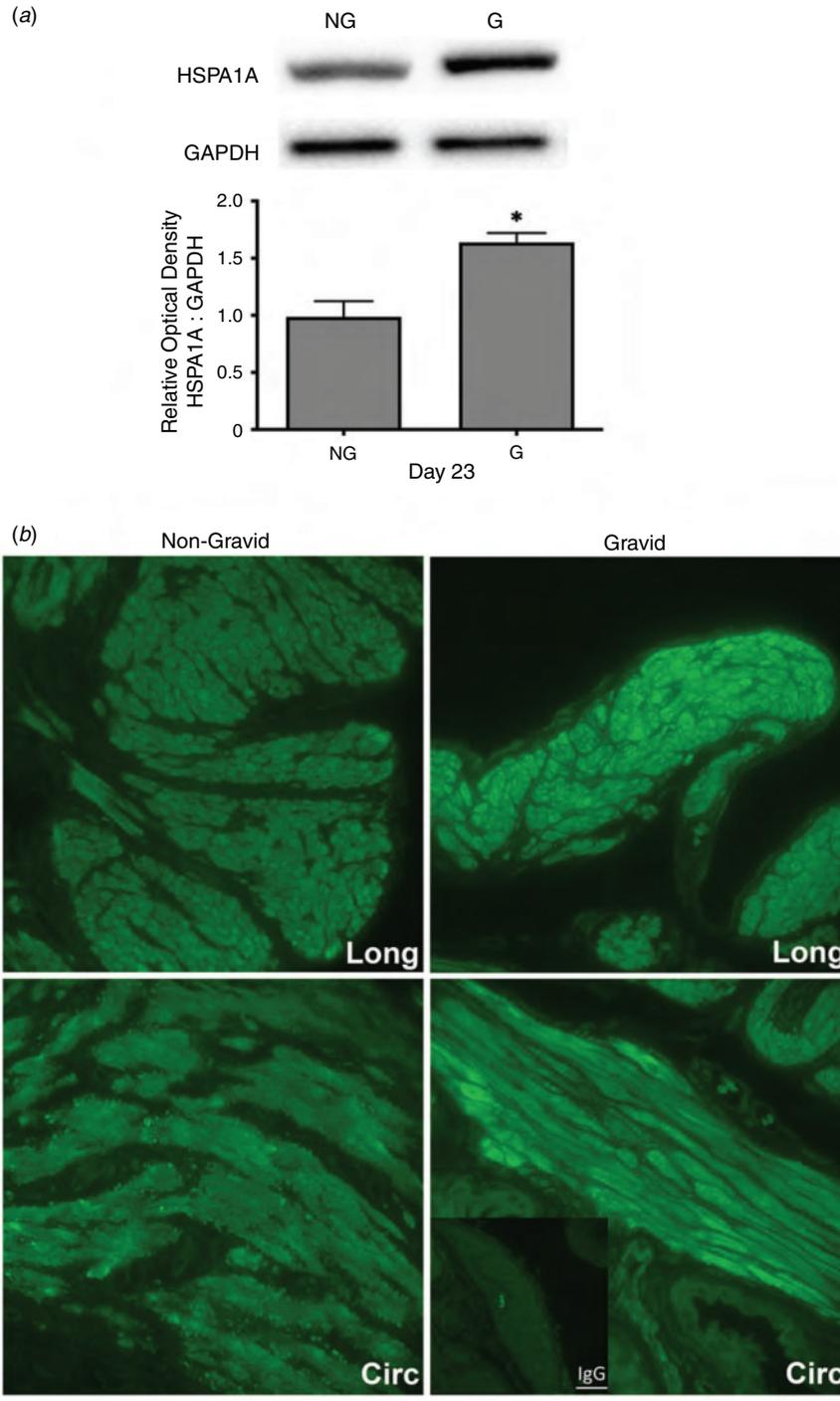
In unilaterally pregnant rats, HSPA1A protein expression in gravid horns on Day 23 of gestation was significantly increased and more immunodetectable *in situ* compared with non-gravid horns. In support of this finding, mechanical distension has also been shown to upregulate HSPA1A expression in the smooth muscle of the urinary bladder and vascular smooth muscle cells (Galvin *et al.* 2002; Metzler *et al.* 2003). Although there were no significant differences in HSPA1A expression or immunostaining on Day 19 of gestation between non-gravid and gravid horns (data not shown), this may just reflect a stress in the gravid horns below the threshold required to induce HSPA1A expression, because it became significantly elevated during gestation after Day 19. The distension stress would peak at Day 23 due to the maximum size of the conceptus. The effect of such a stress on HSPA1A expression is likely complex. Studies using rat and human myometrial cells have demonstrated that distension stress induces inflammation (Shynlova *et al.* 2013; Keelan 2018). Inflammation and oxidative stress are closely interrelated processes that can mutually amplify one another (Hajjar and Gotto 2013). In skeletal and cardiac muscles, as well as in organs such as the liver, oxidative stress was complemented by high levels of HSPA1A (Salo *et al.* 1991). When HSPA1A or HSPA8 were overexpressed in human amnion epithelial cells, interleukin (IL)-8 and Toll-like receptor (TLR) 4 mRNA expression increased, and these effects were abrogated upon respective short interfering RNA targeting (Geng *et al.* 2017). Thus, the

expression of HSPA members in the myometrium may be influenced by the integrated effects of myometrial distension, inflammation and oxidative stress, and may play a role in regulating immune activation within the tissue.

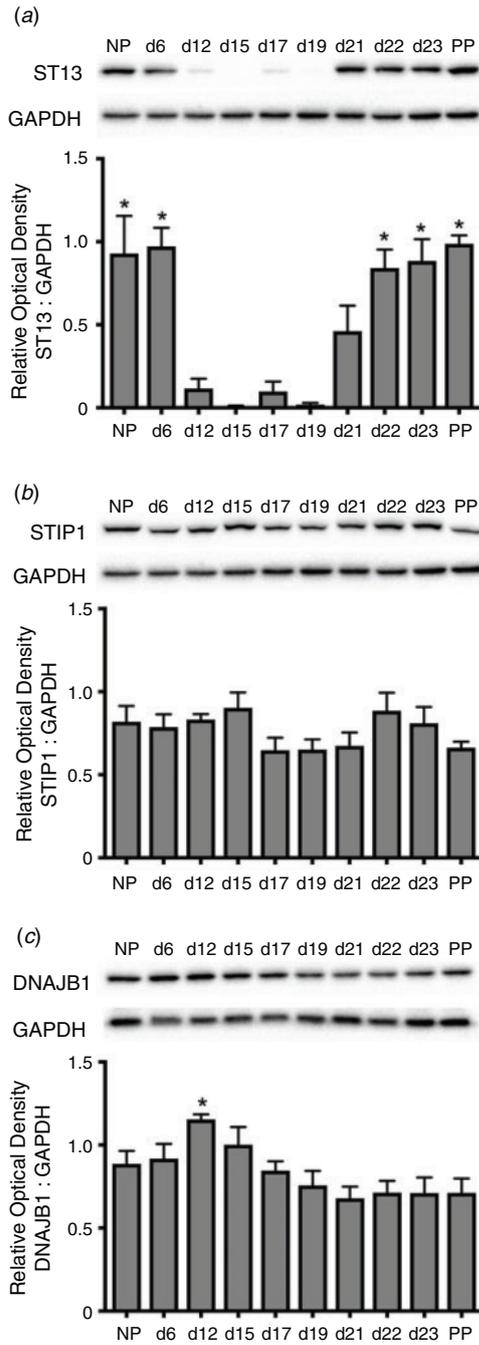
#### Extracellular HSPA1A

From Day 19 of gestation onwards in this study, HSPA1A was frequently detectable in apparent extracellular areas just outside myocyte plasma membranes of both the longitudinal and circular layers of the myometrium. HSPA1A is considered an intracellular protein, but under stressful conditions it is now known to be released into the surrounding environment and circulation (De Maio and Vasquez 2013). HSPs, including HSPA1A, have been found to be released from cells as cargo in EVs (Bausero *et al.* 2005; Clayton *et al.* 2005; Conde-Vancells *et al.* 2008; Vega *et al.* 2008). In fact, HSPA and HSP90 family members are now considered markers of such EVs (Kalluri and LeBleu 2020). EVs produced by pregnancy-associated tissues, including the conceptus, uterus and trophoblast, as well as within the amniotic fluid and serum of pregnant women, have been reported to contain cargo such as small RNAs, mRNA and proteins (Fukushima *et al.* 2005; Asea *et al.* 2008; Stenqvist *et al.* 2013; Burns *et al.* 2016; Bidarimath *et al.* 2017). The roles of extracellular HSPA1A (eHSPA1A) revolve, in particular, around regulation of inflammation, including stimulating proinflammatory cytokine activity (Bausero *et al.* 2005).

In this study, the novel detection of EVs from hTERT-HM cell-conditioned medium that were free of contaminating endoplasmic reticular calnexin, confirmation of HSPA1A as EV cargo from these cells and the immunodetection of eHSPA1A in rat myometrium *in situ* suggest that myometrial cells *in vivo* could release HSPA1A during pregnancy as a cytokine in EVs. HSPA8 and HSPA1A in particular can become inserted into membranes and exist on the cell surface, dependent on membrane fluidity and phospholipid-specific association, enabling



**Fig. 5.** Effect of uterine distension on inducible heat shock protein A1A (HSPA1A) expression in the myometrium during pregnancy. (a) Representative immunoblots of HSPA1A and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression are provided for Day 23 of gestation. Expression of HSPA1A on Day 23 was significantly higher ( $*P < 0.05$ ) in the gravid (G) than non-gravid (NG) horn. Data are the mean  $\pm$  s.e.m. of four independent experiments. (b) Immunofluorescence detection of HSPA1A in both the longitudinal (Long) and circular (Circ) muscle layers of the rat myometrium at Day 23. Detection of HSPA1A was noticeably greater in the gravid than non-gravid horn. Scale bar = 50  $\mu$ m.



**Fig. 6.** Examination of (a) suppressor of tumorigenicity 13 (ST13), (b) stress-induced phosphoprotein 1 (STIP-1) and (c) 40-kDa heat shock protein (DNAJB1) expression in the rat myometrium during pregnancy. Representative immunoblots for each protein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown above the densitometric analyses. (a) ST13 was dynamically expressed in the myometrium during pregnancy. Specifically, expression on Day (d) 6 of gestation remained similar to non-pregnant (NP) expression, and levels at both time points, as well as at Days 12 and 15 and 1 day postpartum (PP) were significantly higher than on Days 12, 15, 17 and 19 of gestation ( $*P < 0.05$ ). (b) STIP-1 was constitutively expressed at high levels in the myometrium throughout pregnancy. (c) DNAJB1 expression showed slightly higher levels at Day 12 of gestation than on Days 19, 21, 22 and 23 and PP ( $*P < 0.05$ ). Data are the mean  $\pm$  s.e.m. of four independent experiments.

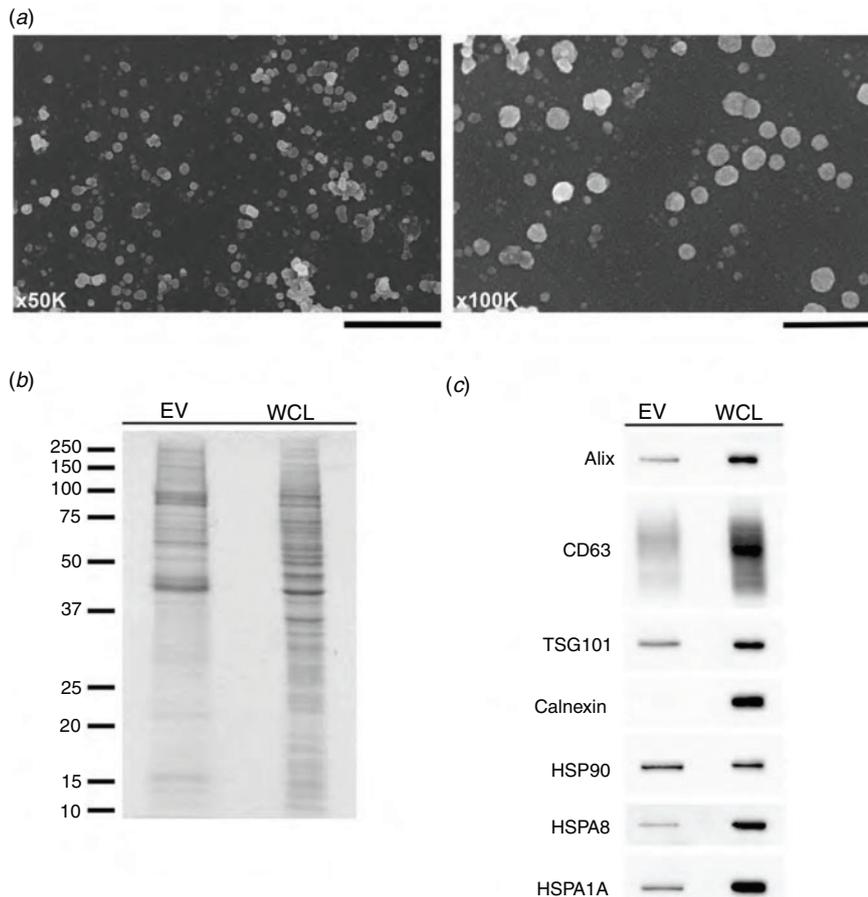
interaction with cell receptors (Ferrarini *et al.* 1992; Multhoff *et al.* 1995; Asea *et al.* 2000; McCallister *et al.* 2016). eHSPA1A is now considered a damage-associated molecular pattern (DAMP) that induces cytokine production and activation of nuclear factor- $\kappa$ B (Asea 2003). Asea *et al.* (2002) reported that eHSPA1A from human embryonic kidney cells bound in a CD14-dependent manner to TLR2 and TLR4 to stimulate the upregulation of tumour necrosis factor- $\alpha$ , IL-1 $\beta$  and IL-6.

The initiation and process of labour have been described as sterile inflammatory events (Shynlova *et al.* 2013). It has been observed that there is a marked increase in immune cell infiltration in pregnancy-associated tissues during labour (Hamilton *et al.* 2012). Leucocytes enhance the effects of proinflammatory cytokines, which, in turn, recruit additional leucocytes. Immune cells, such as macrophages and neutrophils, infiltrate the myometrium and cervix during labour and are implicated in triggering myometrial contractions and inducing cervical ripening via the local release of inflammatory mediators (Osman *et al.* 2003). The release of eHSPA1A from myometrial cells via EVs could result in uptake by additional myometrial cells, the interstitial telocytes of the myometrium that also produce EVs (Cretoiu *et al.* 2013), capillaries and immune cells in the region as part of an intercellular communication network to potentiate immune activation within the myometrium leading to parturition.

Evidence for the detection of eHSPA1A in pregnancy-related tissues or in sera from pregnant women has been reported previously. Molvarec *et al.* (2007) demonstrated a significant positive correlation between gestational age and serum HSPA1A concentrations. Furthermore, it has been reported that HSPA1A serum concentrations were significantly elevated in pregnant women at higher risk of preterm delivery or diagnosed with pre-eclampsia compared with healthy pregnant women (Fukushima *et al.* 2005). Term parturition has also been associated with elevated amniotic fluid concentrations of HSPA1A (Chaiworapongsa *et al.* 2008).

## Conclusion

The present study is the first to demonstrate that HSPA1A expression in the myometrium is significantly upregulated in late pregnancy and labour, localises to both the cytoplasm and nuclei of myocytes and is regulated, in part, by uterine distension. Intracellular HSPA1A could act as part of a chaperone machine to regulate the function of steroid receptors, but novel detection of HSPA1A-laden EVs from human-derived myometrial cells suggests eHSPA1A could be packaged out of myometrial cells as a cytokine to act as a significant mediator of the inflammatory processes and intercellular communication cascades required for parturition. Such dual roles in other cells have led to the designation of HSPA1A as a chaperokine (Asea 2003). Investigations at the molecular level are required to understand the mechanism(s) of HSPA1A action intracellularly and extracellularly within the myometrium. Furthermore, specific assessment of the range of vesicle sizes produced by myometrial-derived hTERT-HM cells and potential size-related functions are warranted.



**Fig. 7.** Assessment of myometrial cell-derived extracellular vesicles (EVs) and their cargo. (a) Scanning electron microscopy examination of samples at magnifications of  $\times 50\,000$  ( $\times 50K$ ; scale bar = 500 nm) and  $\times 100\,000$  ( $\times 100K$ ; scale bar = 250 nm) confirmed the presence of EVs ranging from 20 to 200 nm in diameter. (b) Protein-stained immunoblots demonstrated an enrichment of mid to high molecular weight proteins in EV lysates compared with whole cell lysates (WCL). (c) EV lysates contained the markers apoptosis-linked gene 2-interacting protein X (Alix), cluster of differentiation 63 (CD63) and tumour susceptibility gene 101 (TSG101), as well as 90-kDa heat shock protein (HSP90), heat shock cognate 70-kDa protein (HSPA8) and inducible heat shock protein A1A (HSPA1A), but not the endoplasmic reticular protein calnexin. Representative immunoblots for each protein are shown from four independent experiments.

### Conflicts 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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