Dynamic changes in gene expression and signalling during trophoblast development in the horse

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Abstract

Equine chorionic girdle trophoblast cells play important endocrine and immune functions critical in supporting pregnancy. Very little is known about the genes and pathways that regulate chorionic girdle trophoblast development. Our aim was to identify genes and signalling pathways active in vivo in equine chorionic girdle trophoblast within a critical 7-days window. We exploited the late implantation of the equine conceptus to obtain trophoblast tissue. An Agilent equine 44K microarray was performed using RNA extracted from chorionic girdle and chorion (control) from equine pregnancy days 27, 30, 31 and 34 (n=5), corresponding to the initiation of chorionic girdle trophoblast proliferation, differentiation and migration. Data were analysed using R packages limma and maSigPro, Ingenuity Pathway Analysis and DAVID and verified using qRT-PCR, promoter analysis, western blotting and migration assays. Microarray analysis showed gene expression (absolute log FC >2, FDR-adjusted P <0.05) was rapidly and specifically induced in the chorionic girdle between days 27 and 34 (compared to day 27, day 30 =116, day 31 =317, day 34 =781 genes). Pathway analysis identified 35 pathways modulated during chorionic girdle development (e.g. FGF, integrin, Rho GTPases, MAPK) including pathways that have limited description in mammalian trophoblast (e.g. IL-9, CD40 and CD28 signalling). Rho A and ERK/MAPK activity was confirmed as was a role for transcription factor ELF5 in regulation of the CGB promoter. The purity and accessibility of chorionic girdle trophoblast proved to be a powerful resource to identify candidate genes and pathways involved in early equine placental development.

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Introduction

Mammalian placental development requires a highly regulated series of cellular processes that transform a single layer of trophoblast into a complex membrane that interacts intimately with maternal tissues. Although the anatomical structure of mature mammalian placentae is highly variable between species, the processes that lead to its formation are remarkably similar (Noronha & Antczak 2010, Soncin et al. 2015). Trophoblast cells that give rise to the placenta must undergo a period of rapid proliferation, followed by cell movement, differentiation, migration and in some species also invasion. Once trophoblast penetrate the endometrial epithelium, their immunomodulatory behaviour becomes critical to the prevention of immune-mediated pregnancy loss (Noronha & Antczak 2010, de Mestre et al. 2011, Moffett et al. 2017). These cellular processes are difficult to study in human pregnancy due to the limited availability of tissues at key consecutive developmental stages. Consequently, much work has focused on rodent models of placentation (Underhill & Robins 2016), in vitro models of human pregnancy (Sibley 2017), together with comparative models of placentation (Imakawa et al. 2017) that are both informative for understanding basic mammalian processes as well as being relevant to the species itself. Collectively these studies have revealed a number of genes and signalling pathways involved in placental development (Soncin et al. 2015, Gupta et al. 2016, Haeger et al. 2016) but exactly how important each of these pathways are for the multiple/overlapping processes that are occurring during development and whether these pathways are all active in vivo is less clear.

The early equine conceptus is unique in that it remains in a spherical shape surrounded by a glycoprotein capsule for the first 21 days of gestation. It is only after this capsule is lost that placental development begins in earnest (Allen & Wilsher 2009). Further, the late implantation in the horse (around day 40) means it is remarkably accessible and remains essentially separate to maternal tissues throughout early placental development. This provides distinct
advantages over other species as it allows for isolation of pure populations of trophoblast cells with minimal manipulation (Noronha & Antczak 2010). By day 30 of gestation, the horse placenta is comprised of two main membranes. First, the allantochorion that undergoes rapid growth to form the diffuse epitheliocorial placenta of the horse primarily involved in nutritional exchange and later endocrine support of the pregnancy (Allen & Wilsher 2009). Second, the unique chorionic girdle which gives rise to the endometrial cups that secrete equine chorionic gonadotrophin (eCG) (Antczak et al. 2013) and modulate immunity to foetally derived tissues (de Mestre et al. 2010, 2011). Around day 27 of gestation, the trophoblast cells of the chorionic girdle begin to rapidly proliferate leading to multiple layers of trophoblast cells. Around day 30 of gestation chorionic girdle trophoblast cells receive a signal to terminally differentiate from uninucleate cells to mature eCG-secreting binucleate cells (Cabrera-Sharp et al. 2014) – a process which is close to complete by days 34–36 of gestation. As the trophoblast cells differentiate they acquire a highly invasive phenotype and penetrate the endometrial luminal epithelium and migrate down the glands. Motility then ceases, cells enlarge and form what can be seen as mature endometrial cup trophoblast. The molecular mechanisms that regulate the development of the chorionic girdle, including the co-ordinated processes of proliferation, differentiation, cell movement and cell migration are poorly understood.

We recently identified bone morphogenetic protein (BMP) 4 signalling as a key regulator of trophoblast differentiation in the horse (Cabrera-Sharp et al. 2014). Whilst BMP4 was able to drive terminal differentiation and eCG secretion by the cells, not all cells differentiated in response to the ligand. This suggests other signals are also required to drive chorionic development. Expression of additional growth factor receptors during chorionic girdle development has also been shown (Stewart et al. 1994, 1995, Allen et al. 2007, 2017) although the functional importance and the activity of the associated molecular pathways has not been investigated. Previous studies have used equine-specific microarrays to study gene expression and/or imprinting in the placenta between days 8 and 14 and at day 34 (Klein & Troedsson 2011, Brosnahan et al. 2012, Wang et al. 2013, Iqbal et al. 2014). These studies have found vital insights into the role of interleukin 22 (IL-22) in trophoblast invasive capacity (Brosnahan et al. 2012) and to factors likely to be important in the process of maternal recognition of pregnancy (Klein & Troedsson 2011). An understanding of the gene changes in the chorionic girdle during the time of trophoblast proliferation, differentiation, invasion and induction of eCG expression and secretion would offer key advances in the knowledge of these complex processes.

In this present study, we exploited the late implantation of the equine conceptus to obtain trophoblast tissue between days 27 and 34 of pregnancy from matched mare and stallion pairs. We used whole transcriptome profiling in order to measure and compare gene expression in chorionic girdle trophoblast and adjacent regressing chorion at pregnancy day 27 (initiation of proliferation and prior to differentiation), day 30 (initiation of differentiation), day 31 (consolidation of differentiation and movement of cells) and day 34 (when the majority of the trophoblast cells have terminally differentiated into binucleate eCG-secreting trophoblast and have started to obtain invasive qualities and immunomodulatory capacities). Differentially expressed genes were then identified to determine functions and signalling pathways whose activity was modulated over this critical period of trophoblast development. A selection of genes and pathways were subsequently validated.

Materials and methods

Animals and tissue collection

The study was approved by the Ethics Committee of the Royal Veterinary College and Home Office (PPL70/6944), and all animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 guidelines set by the Home Office, United Kingdom. Five mares (Equus caballus) aged between 3 and 7 years were of Dartmoor or Welsh breed and maintained in a paddock on grass and supplemented with hay over the winter. The reproductive cycle was manipulated using prostaglandin F2, and pregnancies were established as previously described (de Mestre et al. 2011) using semen from two stallions, standard artificial insemination and with ovulation induced using either 1500IU hCG (Chorulon, MSD Animal Health, Milton Keynes, UK) intravenously or 2.1 mg Ovuplant (Dechra Veterinary Products, Shrewsbury, UK) subcutaneously. Ovulation was confirmed (day 0) and then pregnancies were monitored biweekly and on the day of isolation using transrectal ultrasonographic evaluation of the reproductive tract. Only those conceptuses confirmed to have a normal growth rate and normal anatomical development were included in the study. The same mare and stallion pairs (n=5) (Supplementary Table 1 shows exceptions to this, see section on supplementary data given at the end of this article) were used to generate multiple conceptuses that were recovered at day 27, 30, 31 and 34 of pregnancy via nonsurgical uterine lavage as previously described (de Mestre et al. 2008). Additional mare and stallion combinations (n=3) generated conceptus tissue that was subsequently used for qRT-PCR and western blotting. Conceptuses were flushed into sterile phosphate buffered solution supplemented with 2× penicillin–streptomycin (5000U/mL, Invitrogen Gibco) and placed immediately onto ice for transport to the laboratory. Conceptuses were dissected in PBS-2xPenStrep into components, chorionic girdle, chorion, allantochorion, yolk sac and foetus using a dissecting microscope (Zeiss). Dissected tissue was immediately snap-frozen in liquid nitrogen and stored at −80°C until RNA isolation or western blotting was performed.
Isolation and qualitative and quantitative analysis of RNA

Total RNA was isolated from snap-frozen equine conceptus tissue, following homogenisation by QiAshredder (Qiagen), using a Qiagen RNeasy kit (Qiagen) as directed by the manufacturer. RNA was additionally treated with DNase on a column (Qiagen) as described by the manufacturer. RNA concentration and purity was determined by spectrophotometry, using the Nanodrop ND1000 spectrophotometer. The mean 260/280 ratio was 2.10 (range 2.02–2.16). RNA quality was additionally assessed using Agilent 2100 Bioanalyzer. The mean and median RNA integrity number was 9.1 (range 8–9.9) (Supplementary Fig. 1). Samples were stored at -80°C.

Microarray analysis

A 44K probe, single colour, equine Agilent Microarray (cyanine 3-CTP; Agilent Technologies UK Ltd) was carried out by Dr. Lucille Rainbow, at the Centre for Genomic Research, The University of Liverpool. The above described DNase-treated RNA samples were submitted for analysis. Using RNA spike-in kits (Agilent Technologies UK Ltd) and via the manufacturer’s instructions, 50ng of RNA sample was spiked with quality controls and run. cRNA was synthesised and amplified using the low input quick amp labelling kit via the manufacturer’s instructions (Agilent Technologies UK Ltd.). A specific T7 RNA polymerase blend was used to generate fluorescent cRNA via incorporation of Cyanine 3-CTP. cRNA was purified with the RNeasy mini spin column kit (Qiagen), following the manufacturer’s protocol and concentration measured on the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). cRNA was hybridised to the microarray chip using the gene expression hybridisation kit (Agilent Technologies UK Ltd.), using the manufacturer’s instructions and then hybridised for 17 h at 65°C and 10 g using the Tecan HS Pro hybridisation station (Tecan group Ltd, Reading, UK). Microarray chips were washed with wash buffer and scanned by the Agilent DNA microarray scanner G2505C (Agilent Technologies UK Ltd). Samples were subjected to Agilent quality control measures, which all entities passed. Data have been submitted to NCBI Gene Expression Omnibus (GSE113072).

Following Principal Component Analysis (PCA) (Supplementary Fig. 2), a subset of two arrays (out of the total of 40) were removed. These two samples were subsequently shown by qRT-PCR (data not shown) to express significantly aberrant levels of well-known markers of chorionic girdle cells such as chorionic gonadotrophin A (CGA), chorionic gonadotrophin B (CGB) and glial cells missing 1 (GCM1). Pre-processing steps of background correction and between-array normalisation were performed prior to analysis using the software packages R (http://www.r-project.org) and Bioconductor (http://www.bioconductor.org). Differential expression analysis was performed using the R package limma (Ritchie et al. 2015). Pre-processing steps of background correction and between-array normalisation were implemented prior to analysis with differentially expressed genes identified using a linear model fit (lmFit) and an empirical Bayes method (eBayes). Genes with an absolute log fold change >2 and false discovery rate (FDR)-adjusted P<0.05 were considered significant. Cluster gene analysis was performed using maSigPro (Conesa & Nueda 2017) to group genes based on their expression profile. The cut-off criteria used in maSigPro were fold change >2, R2>0.7 and P value of >0.01. DAVID Bioinformatics Resources 6.7 (Huang et al. 2009) functional annotation tool was used to evaluate signalling pathways within gene clusters. Gene lists were input to DAVID, selected to compare against ‘All species’ and gene names defined as ‘Official_gene_symbol’. Output signalling pathways in KEGG and PANTHER databases were considered, with parameters set to include pathways containing >5 genes with P<0.01 (Fishers Exact test).

The normalised intensity data for chorionic girdle tissue only was also imported into Ingenuity Pathway Analysis (IPA) V119043121 software (Qiagen) for analysis of activation of canonical pathways and regulator effect networks. For analysis in IPA, one-way ANOVA (P<0.05) applied across the timecourse was used to identify gene changes and pathway activation within the chorionic girdle. Output data were represented for canonical pathway activation and as summary lists of genes involved in effector networks of interest.

Multiplex qRT-PCR

Eight genes were selected from the array to fit the following criteria: upregulated or downregulated with a fold change 2–5 or >20 in chorionic girdle between day 27 and day 34. As a control, Nuclear RNA Export Factor 1 (NXF1), a gene that remained constant in expression in the chorionic girdle between days 27 and 34, was also assessed. Primers were designed using GeXP eXpress Profile software in accordance to manufacturer instructions (Beckman Coulter, High Wycombe, UK), using mRNA sequences obtains from Ensembl genome browser (http://www.ensembl.org) (Supplementary Table 2). Target-specific reverse transcription and PCR amplification was performed as previously described (Hu et al. 2012, Xie et al. 2012, Staines et al. 2016) and in accordance to manufacturer’s instructions (Beckman Coulter). In brief, a reverse transcription master mix was prepared as detailed in the GeXP starter kit (Beckman Coulter) and performed using a G-storm thermal cycler, using the programme protocol 48°C (1 min), 42°C (60 min), and 95°C (5 min). From this an aliquot was of each reverse transcription reaction was added to GenomeLab kit PCR master mix (Beckman Coulter) and Thermo-Start Taq DNA polymerase (Thermo Fisher Scientific). PCR reaction was again performed using G-storm thermal cycler with a 95°C activation step 10 min, followed by 35 cycles of 94°C (30s), 55°C (30s) and 70°C (60s). Products were separated and quantified using CEQ 8000 Genetic Analysis System and GenomeLab Fragment Analysis software (Beckman Coulter). Genes of interest were normalised against the geo-mean of the two housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase complex, subunit A (SDHA), as previously described (Vandesompele et al. 2002).

Western blotting

Tissues were ground and lysed on ice in 200µL lysis buffer containing: 150 mM sodium chloride (Sigma Aldrich, Haverhill,
UK), 1.0% (v/v) Nonidet P-40 (Sigma Aldrich), 0.5% (w/v) sodium deoxycholate (Sigma Aldrich), 0.1% (w/v) Sodium dodecyl sulphate (Sigma Aldrich), 50 mM Tris, pH 8.0 (Sigma Aldrich), 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich, added at time of use). Protein concentration was determined using Bradford assay (Bio-Rad Laboratories) and read using a Tecan infinite pro 200 plate reader, with Magellan 7 software (Tecan group Ltd.). Samples were stored at −20°C until use.

To assess total and phospho p44/42 extracellular signal regulated kinase (ERK1) protein expression, 50 μg of protein were loaded per well and separated by SDS-PAGE on a 10% (wt/vol) polyacrylamide gel before being transferred to a polyvinylidene difluoride membrane (GE Healthcare Life Sciences) using a Mini-PROTEAN Tetra cell wet transfer unit (Bio-Rad Laboratories). The membranes were blocked in Tris-buffered saline-Tween 20 containing 5% (wt/vol) nonfat milk (Marvel, Premier Foods Group, London, UK) for a total of 3 h. Membranes were incubated overnight at 4°C in a 1:1000 dilution of rabbit anti-human p44/42 total ERK or Rabbit anti-human p44/42 phospho-ERK (Cell Signalling Technology, Leiden, The Netherlands) polyclonal antibodies each in Tris-buffered saline-Tween 20 containing 5% (wt/vol) nonfat milk. Membranes were incubated with a 1:10,000 dilution of goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Sigma) in Tris-buffered saline-Tween 20 containing 5% (wt/vol) nonfat milk. Proteins were visualised by incubating with ECL plus detection reagents (PerkinElmer) and exposed onto Amersham hyperfilm ECL (GE Healthcare Lifesciences). As a loading control, membranes were stripped and reprobed for β-actin using a monoclonal mouse β-actin antibody (Sigma) at a dilution of 1:10,000.

Cloning of ELF5 and transactivation experiments

Full length equine ELF5 was cloned into the pCMV-Myc expression vector for co-transfection experiments. ELF5 was amplified by RT-PCR from equine day 34 chorionic girdle cDNA using primers ELF5F CCATGGT ACCATGATGTGGA CTACGTAGAC and ACGGTCTAGAT CATAGCTTGTCCTCCTGC. The pCMV-Myc vector and ELF5 PCR product were subjected to digest with XbaI and KpnI restriction and gel purified before ligation into pCMV-myc vector for co-transfection experiments. Full length equine ELF5 was cloned into the pCMV-Myc expression vector for co-transfection experiments.

Results

Transcriptomic profile of chorionic girdle between days 27 and 34 of pregnancy

The data reported below used sibling conceptuses obtained at days 27, 30, 31 and 34 of pregnancy over two breeding seasons (total n = 38 samples, n = 4–5 each time point and each tissue). All conceptuses used in the study showed normal growth patterns and had normal placental and foetal anatomy as assessed clinically by transrectal ultrasonography and grossly under a dissecting microscope. For mare stallion pairs that did not generate a full set of conceptuses, half-sibling conceptuses were used (Supplementary Table 1).

The samples grouped well by tissue and gestational age (Supplementary Fig. 2). Analysis of global changes in gene expression revealed that 2207 probes were differentially expressed in the chorionic girdle and chorion across one or more time points or between tissues (Absolute log fold change >2, FDR-adjusted P < 0.05) (Supplementary Table 3). Gene expression in the control tissue (chorion) was remarkably stable throughout the gestation period studied, with only 11
genes differentially expressed in the chorion between days 27 and 34 (Table 1). In contrast, gene expression was rapidly induced in the chorionic girdle over the same time period (Table 1), with a peak of 781 genes differentially expressed in the chorionic girdle between day 27 and day 34. Consistent with a wave of genes that are turned on or off and off again during chorionic girdle development, there was significant overlap in the differential expression of genes at day 30, 31 and 34 (Fig. 1A). Similarly, there was a significant increase in differential gene expression between the chorionic girdle and adjacent chorion between days 30 and 34 (Fig. 1B and Table 1). At day 27, no genes were differentially expressed between chorionic girdle and chorion but this rapidly changed during chorionic girdle development and by day 34 there were 1454 probes representing 754 genes that were differentially regulated between the two tissues.

Validation of genes by qRT-PCR
To validate the microarray data, multiplex qRT-PCR was performed. Eight genes were selected from the array to fit the following criteria: up/downregulated with an absolute fold change between 2 and 5 or >20 in the chorionic girdle between day 27 and day 34. Of the eight genes analysed, six of the expression profiles (kruppel like factor 10 (KLF10), E74-like factor 5 (ELF5), paired box protein 6 (PAX6), SMAD family member 7 (SMAD7), Cytochrome P450 17A1 (CYP17A1) and NXF1) were highly reproducible between the microarray data and multiplex qRT-PCR data (Fig. 2). The remaining two genes (S100 Calcium-Binding Protein A12 (S100A12) and Forkhead Box Protein 1 (FOXP1)) showed very similar patterns of expression in the two tissues, although statistical significance did not always match. The magnitude of the fold change in expression in the chorionic girdle between days differed between assays for some genes, although always remained in the same direction and, with the exception of FOXP1, also continued to fall into the same magnitude grouping used for its selection (2–5 or >20). The control gene, NXF1 did not change in expression in the chorionic girdle at any time point between days 27 and 34. Although the fold change in expression was significantly different between the chorionic girdle and chorion for NXF1, the magnitude was always less than 1.5-fold.

Top upregulated and downregulated gene lists
The 20 top genes that were up/downregulated in the chorionic girdle between day 27 and 34 (absolute fold change >2, \( P<0.05 \)) are listed in Table 2 (up) and Table 3 (down). Of the top 20 upregulated genes at day 30, 15 genes also appeared in the top upregulated gene list for days 31 and/or 34. CGA, CGB, GCM1, MHC Class I, were all in the top 20 upregulated gene list. Interleukin 22, previously shown to be highly upregulated in day 34 chorionic girdle, was not one of the top 20 upregulated genes but was upregulated in day 34 chorionic girdle compared with day 27 chorionic girdle (log fold change 3.94, \( P=0.001 \)). Top upregulated genes included fatty acid-binding protein 4 (FABP4) that peaked in expression at day 31 and transcription factor BCL6, highly upregulated at days 30 through 34. Immune genes, such as CXCL14, also featured in the top 20 list with expression highly induced as early as day 30.

Clustering of genes into expression patterns
Next, the R package SigPro software was used to cluster genes based on their expression patterns across both time and tissues. Nine different gene expression profiles were identified (Fig. 3) each containing between 13 and 374 genes with an R2 regression fit of >0.7. Supplementary Table 4 provides full gene lists for each cluster. For
example, genes included in the gene list for cluster 1 increased in expression in the chorionic girdle (Fig. 3, cluster 1, red) and remained constant in expression in the chorion (green) over the same time period. Genes in clusters 1 and 7 which remained relatively constant in expression in the chorion and gradually increased or decreased in expression in the chorionic girdle between days 27 and 34 (cluster 1 and 7) were identified as genes and components of signalling pathways likely to be involved in cellular growth as either regulators of proliferation and/or differentiation or as markers of the change in the cells differentiation state. Gene lists from these two clusters were input into DAVID functional annotation tool, which identified 37 pathways ($P<0.01$) (Table 4). Pathways of note were epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) signalling (11 and 10 entities respectively), integrin signalling (22 entities) and cytoskeletal regulation by Rho GTPases (10 entities). Genes that showed a similar pattern to clusters 1 and 7 but showed higher expression values by day 30/31 were clustered into groups 4 and 5. Four pathways were enriched in this gene list including genes related to the function of the lysosome (13 genes) and cytoskeletal regulation by Rho GTPases (9 entities). Genes that remained relatively constant in the chorion and rapidly increased in expression in the chorionic girdle at day 34 more consistent with genes associated with the acquisition of an invasive phenotype were also identified (Fig. 3 and Supplementary Table 3, clusters 2 and 6). The pathways represented by clusters 2 and 6 included focal adhesion (16 entities), chemokine signalling (11 entities) and tight junctions (10 entities) (Table 4). Cluster 3 revealed 83 genes that peak at days 30/31 specific to when differentiation is initiated (Supplementary Table 3, cluster 3 gene list). Clusters 3, 8 and 9 were not further analysed in pathway analysis due to relatively low total number of genes on these lists.

**Top regulator effector networks**

Next, IPA was used to further explore the effector networks active during development of the chorionic girdle. Networks in the chorion were not assessed as only 11 genes were differentially expressed in day 34 chorion (compared to day 27) and no genes differed in expression at other time points. The top 5 regulated effector networks at days 30, 31 and 34 (compared with day 27 chorionic girdle) are listed in Table 5. Activation scores were all high, suggesting that gene expression data is highly supportive of the networks identified. Consistent with key cellular processes known to occur during chorionic girdle development, two cellular processes were highly represented in these lists; cell movement/cell migration/invasion (6/15 networks) and proliferation/differentiation (5/15 networks). Cell movement and migration pathways demonstrated an increase in activation score over time from 8.485/6.062 at day 30 to 11.25 at day 31 and scores of 8.014 and 9.865 for migratory pathways at day 34. By day 31, cell cycle regulation and S phase checkpoint control (activation score 9.925) were processes induced in trophoblast and this continues to day 34. Based on the directionality of the expression change in the microarray data, a number of upstream regulators of these effector networks were identified and are also listed in Table 5.

**Top canonical pathways**

IPA was also used to assess the activation of canonical pathways in the chorionic girdle between days 27 and 34 of pregnancy using the full gene list. There were
Regulation of chorionic girdle development

Dynamic changes in predicted pathway activation in the chorionic girdle during development (Fig. 4). Out of a total of 533 pathways stored in the IPA software at the time of analysis, 35 pathways had an activation z-score of >2 or <−2 (P<0.05) at one or more time points during chorionic girdle development (Fig. 4). These included 16 pathways known to be associated with cell movement, 12 pathways known to be involved in cellular immune responses and 12 pathways associated with cell cycle regulation and/or differentiation (Fig. 4). There was a predominance of movement pathways related to signalling by Rho GTPases including activation of RhoA, regulation of actin-based motility by Rho and inhibition of the inhibitory signal, RhoGDI signalling (Fig. 4). Cell cycle pathways with positive activation scores included ILK signalling, aryl hydrocarbon receptor signalling (which peaked at day 30 around initiation of differentiation) and TWEAK signalling.

Validation of genes and pathways

Next, in order to further explore the activity of the genes and pathways identified above, one gene and two pathways were selected for further investigation.

ELF5 transactivates the CGB promoter

Ets family transcription factor ELF5 was selected from the genes confirmed by qRT-PCR for further investigation due to its temporal expression profile that suggested a possible role in regulation of trophoblast development. RT-PCR amplification of ELF5 and ELF4 (a related family member) genes in equine chorionic girdle and control chorion tissues from gestational ages 27 to 34 days (Fig. 5A) showed that ELF5 was expressed in the chorionic girdle and chorion at all time points, with evidently higher expression in the chorionic girdle compared to the chorion. Gene expression of ELF4 was not detectable in any equine conceptus tissues tested, regardless of gestational age. Expression in control spleen tissue demonstrated successful amplification of the ELF4 gene (Fig. 5A). Western blot analysis confirmed the presence of an approximately 37 kDa protein in the chorionic girdle similar to the predicted size of ELF5 (31 kDa) (Supplementary Fig. 3).

This expression profile in the chorionic girdle combined with bioinformatics analysis of the CG/LH promoter that revealed 24 predicted ELF5-binding sites (data not shown) led us to hypothesise that ELF5 may play a role in inducing CG/LHB expression, a key gene induced during differentiation of chorionic girdle cells and the rate-limiting subunit required for eCG production (de Mestre et al. 2009). To explore this further, we used the expression data generated on the microarray to determine if an association existed between CG/LHB and ELF5 expression in all chorion and chorionic tissues (Fig. 5B). ELF5 and CG/LHB mRNA expression in individual chorionic girdle and chorion tissues was strongly positively correlated (R²=0.80, P<0.0001).

To determine whether activity of the CG/LHB promoter was driven by ELF5, co-transfections were carried out in COS7 cells using 250 ng of four truncated CG/LHB promoter inserts (data not shown) led us to hypothesise that ELF5 may play a role in inducing CG/LHB expression, a key gene induced during differentiation of chorionic girdle cells and the rate-limiting subunit required for eCG production (de Mestre et al. 2009). To explore this further, we used the expression data generated on the microarray to determine if an association existed between CG/LHB and ELF5 expression in all chorion and chorionic tissues (Fig. 5B). ELF5 and CG/LHB mRNA expression in individual chorionic girdle and chorion tissues was strongly positively correlated (R²=0.80, P<0.0001).

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Table 2  Top 20 upregulated genes in the chorionic girdle at day 30, 31 and 34 all compared to day 27 chorionic girdle.

| Gene symbol | Gene function                                | LogFC day 30 | LogFC day 31 | LogFC day 34 |
|-------------|---------------------------------------------|--------------|--------------|--------------|
| FABP4       | Fatty acid binding protein                   | 5.81310***   | 7.12020****  | 5.48164****  |
| CG          | Glycoprotein hormone alpha subunit           | 5.24752**    | 6.85655***   | 8.54458****  |
| Unamed^d    | Unknown                                     | 4.62192**    |              |              |
| BCL6        | Transcription factor                         | 4.28718***   | 5.05434****  | 5.70755****  |
| SBSPON      | Scavenger receptor activity                  | 4.14721**    | 5.04306****  | 5.67806****  |
| ENSECAT0000001796 | Ucharacterised protein            | 3.61386**    | 4.59892****  |              |
| FFA2        | G-protein-coupled receptor                   | 3.36892*     | 5.03815****  | 6.84478****  |
| FGF7        | Epithelial cell-specific growth factor       | 3.35574***   | 4.99731****  |              |
| Unamed^h    | Unknown                                     | 3.27125**    | 4.00893****  | 4.90052****  |
| PSMB9       | Proteasome subunit                          | 3.21874**    |              | 5.09380****  |
| ENSECAT00000015366 | Ucharacterised protein            | 2.91600***   | 3.80865****  |              |
| PLAC9       | Placenta specific gene                       | 2.76454**    | 4.03647****  |              |
| CXCL14      | Cytokine                                    | 2.58010*     | 3.43442****  | 5.48339****  |
| CCNB1       | Regulatory protein involved in mitosis       | 2.77803**    |              |              |
| ARG2        | Arginase activity                            | 2.64996**    | 3.27003****  |              |
| MYBL2       | Transcription factor                         | 2.66493**    |              |              |
| RRM2        | Ribonucleotide reductase                     | 2.64996**    | 3.27003****  |              |
| Unamed^d    | Unknown                                     | 2.58010*     | 3.43442****  | 5.48339****  |
| SERPINE2    | Inhibitor of serine protease                 | 2.52432*     | 3.32343****  | 5.53857****  |
| GCM1        | Transcription factor                         | 2.44777*     | 3.51574****  |              |
| Unamed^d    | Unknown                                     | 4.93379**    |              |              |
| Unamed^d    | Unknown                                     | 3.51056**    | 5.97793****  |              |
| FAM227B     | Transcription factor                         | 3.31251****  |              |              |
| MHC Class 1 | Central role in the immune system            | 3.23287****  |              |              |
| TAF4B       | Selective coactivator of transcription       | 3.05323****  |              |              |
| TCN1        | Vitamin B-12 binding protein                 | 8.97029****  |              |              |
| LAMB3       | Basement membrane protein                    | 7.03224****  |              |              |
| Unamed^d    | Unknown                                     | 6.30324****  |              |              |
| ENSECAT0000001317 | Known protein coding          | 6.09937****  |              |              |
| Unamed^d    | Unknown                                     | 5.95520****  |              |              |
| ITGA2       | Transmembrane receptor subunit               | 5.79772****  |              |              |
| CADM1       | Cell adhesion molecule                       | 5.30317****  |              |              |
| Unamed^b    | Unknown                                     | 4.92632****  |              |              |
| Unamed^d    | Unknown                                     | 4.89318****  |              |              |
| CGB         | Chorionic gonadotrophin B-subunit            | 6.52371****  |              |              |

Of the top 20 upregulated genes at day 31, 15 also appeared on the list for day 30. Of the top 20 genes upregulated at day 34, 10 genes appeared on the lists for day 30 or 31.

*P < 0.05; **P < 0.01; ***P < 0.001; "unamed with Ensembl number; "Equine placenta cDNA Library Equus caballus cDNA clone HL02016B1B10, mRNA sequence (DN509596); "Equine Articular Cartilage cDNA Library Equus caballus cDNA clone CT020024A10D12, CX599557; "Equine placenta cDNA Library Equus caballus cDNA clone HL02016B1B10, mRNA sequence (DN509596); "Equine placenta cDNA Library Equus caballus cDNA clone HL02016B1B10, mRNA sequence (DN509596); "Equine placenta cDNA Library Equus caballus cDNA clone HL02016B1B10, mRNA sequence (DN509596); "Equine placenta cDNA Library Equus caballus cDNA clone HL020001000 PLATE_E04_29_023, mRNA sequence (DN507748); "Equine Articular Cartilage cDNA Library Equus caballus cDNA clone CT020021B10E08, mRNA sequence (CX598679); "Equus caballus contig07293.EqcaPBMC mRNA sequence (JL622886); "Equus caballus contig05471.EqcaPBMC mRNA sequence (JL621232); "Equine placenta cDNA Library Equus caballus cDNA clone HL02014A2G04, mRNA sequence (DN508969)."
Regulation of chorionic girdle development

4.19061**** 5.23593**** 3.25615**** 2

Reversible hydration of CO

Unknown

Actin filament binding

Uncharacterised protein

Ensembl number listed for unnamed genes;

LogFC day 30

LogFC day 31

LogFC day 34

2.89347**

4.73441****

3.42092****

Fig. 7B

Unknown

LogFC day 30

LogFC day 31

LogFC day 34

2.51741****

4.66107****

3.89842****

Fig. 6C

Bifunctional enzyme

G protein-coupled receptor

Enzyme for prostaglandin synthesis

Golgi localised protein

Reversible hydration of CO

SRF

There

Enzyme, retinol dehydrogenase

Enzyme, cytokine p450

3.54138****

3.19614****

Uncharacterised protein

Uncharacterised protein

2.83819****

4.55809**

3.00171****

P

LogFC day 34

LogFC day 31

LogFC day 30

5.52317****

2.54894****

5.52317****

Equine placenta cDNA Library

Regulation of actin cytoskeleton

Transmembrane folate transporter

Equine Articular Cartilage cDNA Library

Gene symbol

Gene function

Table 3 Top 20 downregulated genes in the chorionic girdle at day 30, 31 and 34 all compared to day 27 chorionic girdle.

| Gene symbol | Gene function | LogFC day 30 | LogFC day 31 | LogFC day 34 |
|-------------|---------------|--------------|--------------|--------------|
| FABP1       | Fatty acid binding protein | 3.42092****  | 3.98743****  | 4.98492****  |
| GPR162      | G protein-coupled receptor  | 3.41190****  | 4.19061****  | 4.88511****  |
| PTGES2      | Enzyme for prostaglandin synthesis | 3.33766****  | 4.55809**   | 4.92065**   |
| RDH16L      | Enzyme, retinol dehydrogenase | 3.26851****  | 5.10370***  | 6.14734***  |
| IL1B        | Cytokine | 3.25615****  | 4.18707***  | 6.49047***  |
| Unameda     | Unknown    | 3.19614****  | 4.21925****  | 5.01506****  |
| Unamedb     | Unknown    | 3.16932****  | 4.21925****  | 5.01506****  |
| ENSECAT00000022537 | Uncharacterised protein | 3.15012****  | 4.12701****  | 5.00712****  |
| ENSECAT0000007031 | Uncharacterised protein | 3.00171****  | 4.12701****  | 5.00712****  |
| ACTG2       | Cytoskeletal protein | 3.00165****  | 4.12701****  | 5.00712****  |
| FMO1        | Oxioreductase | 2.89347**   | 3.89424****  | 4.87058****  |
| CA7         | Reversible hydration of CO₂ | 2.83819****  | 3.84536****  | 4.87058****  |
| HSD3B1      | Bi functional enzyme | 2.75848****  | 3.84536****  | 4.87058****  |
| ENSECAT00000019764 | Uncharacterised protein | 2.63330****  | 3.64952****  | 4.66107****  |
| AP1L1       | membrane-associated glycoprotein | 2.61260****  | 4.56274****  | 4.66107****  |
| ENSECAT00000020332 | Uncharacterised protein | 2.54894****  | 3.64952****  | 4.66107****  |
| LGMN        | Enzyme, cysteine protease | 2.52946****  | 4.56274****  | 4.66107****  |
| MT          | Golgi localised protein | 2.51741****  | 3.64952****  | 4.66107****  |
| ENSECAT00000021165 | Uncharacterised protein | 2.50448****  | 3.54138****  | 4.89858****  |
| CYP17A1     | Enzyme, cytokine p450 | 2.50254****  | 3.70293****  | 4.89858****  |
| Unamedc     | Unknown    | 4.11917****  | 5.72999****  | 7.23593****  |
| TAGLN3      | Actin filament binding | 4.11782****  | 5.72999****  | 7.23593****  |
| LHFP1       | Transmembrane protein | 4.01210****  | 5.24291****  | 7.23593****  |
| ENSECAT0000024713 | Uncharacterised protein | 3.74453****  | 5.23393****  | 7.23593****  |
| CPVL        | Carboxypeptidase | 3.73216****  | 5.23393****  | 7.23593****  |
| PLXNB1      | Regulation of actin cytoskeleton | 3.64882****  | 5.23393****  | 7.23593****  |
| ENSECAT00000016216 | Uncharacterised protein | 3.64030****  | 4.73441****  | 7.23593****  |
| ENSECAT0000006927 | Uncharacterised protein | 3.64030****  | 4.73441****  | 7.23593****  |
| SLC46A1     | Transmembrane folate transporter | 2.50254****  | 5.05205****  | 7.23593****  |
| Unamedd     | Unknown    | 5.02691****  | 5.05205****  | 7.23593****  |
| Unamedd     | Unknown    | 4.78917****  | 5.05205****  | 7.23593****  |
| CA4         | Reversible hydration of CO₂ | 4.69078****  | 5.72999****  | 7.23593****  |
| PL2A2G10    | Enzyme, phospholipase | 4.57344****  | 5.72999****  | 7.23593****  |

Of the top 20 upregulated genes at day 31, 16 also appeared on the list for day 30. Of the top 20 genes upregulated at day 34, 10 genes appeared on the lists for day 30 or 31.

*R<0.05; **R<0.01; ***R<0.001; ENSEmb number listed for unnamed genes; Unamed symbol with Ensembl number; ENSEmb number with unnamed genes; OFequine placenta cDNA Library Equus caballus cDNA clone HL0200100000_PLATE_G06_47_050, mRNA sequence (DN508408); Equine placenta cDNA Library Equus caballus cDNA clone HL020121A2F04, mRNA sequence (DN510935); Equine placenta cDNA Library Equus caballus cDNA clone HL0201981D12, mRNA sequence (DN510457); Equine placenta cDNA Library Equus caballus cDNA clone HL0200200000_PLATE_G01_7_004, mRNA sequence (DN507842); Equine Articular Cartilage cDNA Library Equus caballus cDNA clone CT02041A1D02, mRNA sequence (CX65310).

Factors, serum response factor (SRF) and CRE-binding protein 1 (CREB1) in the chorionic girdle. Correlating with the increase in ERK1/2 activity at day 30, SRF and CREB1 expression significantly increased at day 31 in the chorionic girdle when compared to day 27 (Fig. 6C).

RhoA is involved in migration of equine chorionic girdle trophoblast

A number of pathways related to Rho signalling were identified in Fig. 4. In order to further explore the functionality of these pathways, primary day 34 equine trophoblast cells were cultured in the presence or absence of Rho inhibitor Rhosin (50 µM) and trophoblast migration assessed using a scratch assay (Fig. 7A). There was no change in the morphology of the cells or evidence of cell death following treatment with Rhosin or ethanol (control). Treatment of chorionic girdle trophoblast with 50 µM Rhosin resulted in a significant reduction in trophoblast migration into the scratch compared to ethanol control treated cells (P<0.001) (n=8, 4 readings per scratch) (Fig. 7B).

Discussion

Chorionic girdle development requires a number of co-ordinated processes that must occur in a timely and controlled manner. Establishing the global gene expression profile for genetically paired conceptuses over a 7-day period of pregnancy (day 27–34) has provided a powerful insight into the genetic landscape of the trophoblast cells as they terminally differentiated from uninucleate trophoblast into eCG-secreting binucleate trophoblast with an invasive phenotype. Fluctuations in gene expression were both dynamic and very specific to the CG whilst the adjacent chorion...
had remarkably stable gene expression over the same time period. The abundant genes and pathways identified were highly consistent across laboratory and bioinformatic approaches, with three of these, ELF5 function, ERK1/2 activity and Rho A function, further confirmed in functional assays.

Initial PCA visualisation of grouping of the tissues demonstrated distinct gene expression profiles in the chorionic girdle at all time points assessed, whilst in contrast, chorion tissues from all time points were genetically identical. In total, only 11 genes were differentially expressed in the control tissue, chorion compared with 1625 in the chorionic girdle. Although chorionic girdle trophoblast at day 30/31 are still primarily uninucleate trophoblast, the gene profile already had substantially shifted (116 and 317 genes respectively) when compared to day 27 cells. This corresponds to the time at which a large proportion of chorionic girdle trophoblasts rapidly proliferate and undergo differentiation to become terminally differentiated binucleate trophoblast cells and is also the period immediately prior to the significant induction of eCG expression (de Mestre et al. 2009, Read et al. 2018).

The genes and pathways switched on/off in this early period in girdle development will provide important clues to the initiators of proliferation, differentiation and eCG expression. For example, fatty acid-binding protein FABP4, a gene known to regulate both proliferation and differentiation genes in human trophoblast cell line HTR8/SVneo (Basak et al. 2018), was found to peak in expression at day 31. C-X-C Motif Chemokine Ligand 14 (CXCL14) expressed in human villous cytotrophoblast (Kuang et al. 2009) and known to negatively regulate trophoblast invasion was highly induced by day 30. It is plausible that CXCL14 is playing a similar role in the chorionic girdle, preventing premature invasion of early forming binucleate cells into the endometrium, which usually does not take place until day 34–38.

In order to further establish that changes in gene expression observed in microarray analysis were truly representative of the biology of the trophoblast cells eight genes were verified by qRT-PCR and all demonstrated the same expression pattern to that of the microarray. A small number of genes shown previously to be expressed in day 34 chorionic girdle (Bacon et al. 2002, de Mestre et al. 2009, Brosnahan et al. 2012) featured on gene and signalling pathway lists providing further confirmation of the validity of the large dataset. For example, gene cluster analysis identified the SMAD1/5 arm of the TGFβ signalling pathway to contain multiple differentially regulated genes in the chorionic girdle, including BMP4, its receptor bone morphogenic protein receptor 2 (BMPRII) and SMAD1/5, all consistent with a known function of this pathway in equine trophoblast (Cabrera-Sharp et al. 2014). EGF signalling in chorionic girdle cells as measured by canonical pathway analysis increased in activity in the chorionic girdle supporting previously reported expression profiles of EGF and EGF receptors in the chorionic girdle (Stewart et al. 1994, Allen et al. 2017). The function of EGF in the chorionic girdle is not known but in human trophoblast, EGF has been shown to promote proliferation through activation of AKT and ERK1/2 signalling downstream of EGFR (Fock et al. 2015, Costa et al. 2016). The ERK/MAPK signalling pathway was confirmed here to be functional in chorionic girdle trophoblasts, increasing in activity at day 30 of pregnancy when compared to day 27. It is therefore likely that EGF signalling acting via ERK1/2 is indeed active in the chorionic girdle.

Phosphorylation of p44/p42 and nuclear translocation is thought to be important for cell cycle re-entry into S phase in response to mitogenic activators (Brunet et al. 1999). S phase checkpoint effector networks are identified at days 31 and 34 here, so it is possible that the MAPK/ERK signalling pathway is crucial for this regulation. Other described roles for the MAPK/
ERK pathway include regulation of cellular growth differentiation (Brunet et al. 1999, Dumaz & Marais 2005). Due to the ubiquitous nature of the MAPK/ERK pathway, it is likely that it plays roles in multiple cellular processes during chorionic girdle development, perhaps activating the cell cycle at day 30-31 and later differentiation at day 31-34.

Fibroblast growth factor 7 (FGF7), a growth factor expressed by bovine immature trophoblast giant cells (Pfarrer et al. 2006), was significantly upregulated.

| Term                                                | No. of genes | P value     |
|-----------------------------------------------------|--------------|-------------|
| Cluster 3 Spliceosome                                | 5            | 0.00016     |
| Clusters 4 and 5                                    |              |             |
| Lysosome                                            | 13           | 0.00110     |
| Cytoskeletal regulation by Rho GTPase               | 9            | 0.00610     |
| Pathogenic *Escherichia coli* infection             | 8            | 0.00230     |
| Ether lipid metabolism                              | 6            | 0.00290     |
| Clusters 1 and 7 Pathways                          |              |             |
| in cancer                                           | 22           | 0.00002     |
| Integrin signalling pathway                         | 22           | 0.00000     |
| Inflammation mediated by chemokine and cytokine signalling pathway | 21           | 0.00007     |
| Focal adhesion                                      | 19           | 0.00000     |
| Regulation of actin cytoskeleton                    | 17           | 0.00003     |
| Angiogenesis                                        | 14           | 0.00320     |
| PDGF signalling pathway                             | 13           | 0.00140     |
| Chemokine signalling pathway                        | 12           | 0.00260     |
| Neurotrophin signalling pathway                     | 12           | 0.00006     |
| EGF signalling pathway                              | 11           | 0.00180     |
| Cytoskeletal regulation by Rho GTPase               | 10           | 0.00083     |
| VEGF signalling pathway                             | 10           | 0.00005     |
| Small cell lung cancer                              | 10           | 0.00004     |
| Renal cell carcinoma                                | 10           | 0.00001     |
| Tight junction                                      | 9            | 0.00660     |
| Endothelin signalling pathway                       | 9            | 0.00110     |
| Prostate cancer                                     | 9            | 0.00038     |
| Chronic myeloid leukaemia                           | 9            | 0.00010     |
| T cell receptor signalling pathway                  | 8            | 0.00580     |
| Fc gamma R-mediated phagocytosis                    | 8            | 0.00260     |
| Insulin signalling pathway                          | 8            | 0.00210     |
| ErbB signalling pathway                             | 7            | 0.00620     |
| Progesterone mediated oocyte maturation             | 7            | 0.00570     |
| ECM-receptor interaction                            | 7            | 0.00510     |
| Colorectal cancer                                   | 7            | 0.00510     |
| Fc epsilon RI signalling pathway                    | 7            | 0.00340     |
| Pancreatic cancer                                   | 7            | 0.00210     |
| Melanoma                                            | 8            | 0.00200     |
| Glioma                                              | 7            | 0.00097     |
| Acute myeloid leukaemia                             | 7            | 0.00059     |
| Endometrial cancer                                  | 7            | 0.00005     |
| Valine, leucine and isoleucine degradation          | 7            | 0.00010     |
| p53 pathway feedback loops 2                        | 6            | 0.00750     |
| Non-small cell lung cancer                          | 6            | 0.00230     |
| Bladder cancer                                      | 6            | 0.00059     |
| Glycerolipid metabolism                            | 5            | 0.00530     |
| Thyroid cancer                                      | 5            | 0.00071     |
| Clusters 2 and 6                                    |              |             |
| Focal adhesion                                      | 16           | 0.00001     |
| Chemokine signalling pathway                        | 11           | 0.00401     |
| Adherens junction                                   | 10           | 0.00000     |
| Tight junction                                      | 10           | 0.00110     |
| Histamine H1 receptor mediated signalling pathway   | 6            | 0.00076     |
| Vibrio cholera infection                            | 5            | 0.00950     |
| GABA-B receptor II signalling                      | 5            | 0.00350     |

The genes identified in maSigPro clusters 3, 4 and 5, 1 and 7 and 2 and 6 were mapped onto pathways in the KEGG and PANTHER databases. Pathways containing a minimum of five cluster genes (P < 0.01) were considered biologically relevant. Shown are the pathway identifiers, the number of cluster genes present in the pathway and the P value (Fishers Exact).
between day 27 and 34, as was the related family member fibroblast growth factor-binding protein 1 (FGFBP1). Previous studies have reported fibroblast growth factor 2 (FGF2), its receptors (fibroblast growth factor receptor 1-4 (FGFR1-4)) and FGF-binding protein 2 to be expressed in equine trophoblast earlier in pregnancy between days 14 and 28 (de Ruijter-Villani et al. 2011). Supportive of a possible autocrine function for FGF7 in the chorionic girdle, FGF signalling was identified in gene cluster analysis with 9 FGF regulated genes found to be induced/repressed between days 27 and 34 and downstream ERK1/2 activity confirmed in chorionic girdle by western blot. This is consistent with other species, where FGF proteins are thought to play a key role in trophoblast development including hCG production (Jeong et al. 2016) via signalling through ERK (Taniguchi et al. 2000, Yang et al. 2011, Kunath et al. 2014). The role for FGF signalling in the equine placenta remains unknown, but data here suggest an autocrine function for FGF signalling in trophoblast development in the chorionic girdle. Based on its high expression pattern, it is plausible that it may also act in a paracrine manner on adjacent tissues such as the allantochorion thus co-ordinating chorionic girdle development and implantation.

ELF5 mRNA expression was found to be one of the most markedly induced genes between days 27 and 34 with maximal expression at day 34. Transactivation studies demonstrated that ELF5 was able to drive the CG/LHB promoter between the regions of 335 and 2500 bp relative to the translation start codon suggesting it may play a role in biochemical differentiation of trophoblast. The large number of potential ELF5-binding sites in this region suggests that ELF5 may drive promoter activity by acting directly to multiple sites in the distal promoter, although further work is required. ELF5 was considered widely considered to be a ‘gatekeeper’ of stem cell status in mouse studies (Hemberger et al. 2010, Pearton et al. 2011, 2014). More recently, work suggests that ELF5s function changes in a concentration-dependent manner with high levels of ELF5 triggering trophoblast differentiation (Latos et al. 2015) achieved through an interaction with transcription factor AP-2 gamma (TFAP2C). Interestingly, we did detect ELF5 transcript in day 27 chorionic girdle, albeit at much lower levels (data not shown). Therefore, it is plausible

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Table 5  Top 5 effector networks and associated upstream regulators identified using IPA.

| Day of gestation | Top upregulated upstream regulators (vs D27 ChG) | Functions of regulated effector networks | Consistency score |
|------------------|-------------------------------------------------|------------------------------------------|-------------------|
| D30              | 1. GLIS2, IgG, IHH, MAP2K1, MAP2K1/2, MAP2K3, miR141-3p, PTHLH | Cell movement of tumour cells | 8.485 |
|                  | 2. Akt, CTNNB1, ERK, ETV4, Histone H3, PRKCZ | Failure of kidney, glomerulosclerosis | 7.778 |
|                  | 3. ETV4, PML, SDCBP, TBX3, TFEB | Accumulation of lipid, cell movement of tumour cells, blood vessel development | 6.306 |
|                  | 4. ETV4, SP11, TLR4 | Cell movement of tumour cells, fibrosis of kidney, urinary system morphology | 6.062 |
|                  | 5. ELF5, EP400, LYL1, RAPF1, TBX2, TFDP1 | Size of animal, size of embryo | 5.277 |
| D31              | 1. BGN, ETV4, ETV5, GLIS1, HNRNPAB, MAP2K1/2, PLCD1, PTHLH, SOCS1, SRC (family) | Migration of cancer cells | 11.250 |
|                  | 2. AHR, BMIP3L, CCND1, E2F2, E2F6, EIF4G1, let-7, PPM1D, RBL1, STUB1, TBX2 | Repair of DNA, S phase checkpoint control | 9.922 |
|                  | 3. ELF5, EP400, Pka, TBX2, TFDP1 | Proliferation of heart cells, size of embryo, quantity of brain cells | 4.750 |
|                  | 4. CDKN1A, E2F1, HDAC1, let-7, mir-10, miR16-5p, Rb, TBX2 | S phase checkpoint control | 4.596 |
|                  | 5. HNRNPAB, MAP3KB, NOS2, POUSF1, SATB1, SPDEF | Progression of tumour | 3.273 |
| D34              | 1. APC, ETV4, ETV5, GLIS2, MAP3K8, PLCD1, SPARC, WISP2 | Cell movement of lymphocytes, migration of cancer cells | 10.800 |
|                  | 2. BMP15, BMPR1A, HNRNPAB, PLA2G5, PLB | Angiogenesis, cell movement of lymphocytes, colony formation of cells movement of cancer cells, proliferation of muscle cells | 9.865 |
|                  | 3. GLIS2, MAP2K1/2, PLCD1, SMAD3, SRC family, TGFB3, WISP2 | Migration of tumour cells | 8.014 |
|                  | 4. CCND1, E2F2, E2F6, EIF4G1, let-7, PPM1D, RBL1, STUB1, TBX2 | Repair of DNA, S phase checkpoint control | 7.649 |
|                  | 5. Akt, CDKN1A, E2F1, FGF2, HDAC1, let-7, mir-10, miR125b-5p, miR16-5p, PTEN, Rb, TBX2 | S phase checkpoint control | 5.303 |

The top 5 upregulated regulator effect networks at each time point, as compared to day 27 chorionic girdle are shown. Identified upstream regulators and their downstream cellular effects are shown, with consistency score for the pathway (z-score >2, P<0.05, IPA).
Figure 4 Activation of canonical pathways in the chorionic girdle during day 27–34 of pregnancy. IPA was used to identify activation or repression of canonical pathways in the chorionic girdle between days 27 and 34 of gestation. All pathways identified by the programme to have an activation (z-score) score of > or < 2 and $P < 0.05$ are shown with green indicating activation and red inactivation. Pathways were grouped according to known functions.
that the chorionic girdle trophoblast utilise ELF5 in a similar manner to mouse cells, with low levels that ELF5 playing a role in maintaining the stem cell status of progenitor chorionic girdle cells (supported here in the pathway analysis which show loss of stem cell pathways between day 27 and 34) and high levels of ELF5 at day 34 driving the switch to differentiation and induction of CG/LHB expression.

Proliferation pathways consistently arose in regulator effect networks at all time points and were represented in canonical pathway analysis by pathways, such as endothelin 1 signalling and cyclin dependent kinase 5 (CDK5) signalling. The kinetics of these pathways suggest a reduced, yet ongoing requirement for trophoblast proliferation throughout chorionic girdle development. The upstream regulator EP400 was identified as a top upstream regulator at days 31 and 34 of pregnancy and is known to play a role in cellular proliferation in other cells types (Mattera et al. 2009). The majority of other signalling data suggests repression of proliferation signalling pathways. Endothelin signalling in cancer cells is known to play diverse roles in proliferation, apoptosis and migration (Rosano et al. 2013) and TNF-related weak inducer of apoptosis (TWEAK) signalling is well defined to promote proliferation of cardiomyocytes (Novoyatleva et al. 2010). It is likely that pathway suppression represents a continuous but proportionally decreasing population of proliferating cells, as increasing numbers of chorionic girdle trophoblasts terminally differentiate, losing their proliferative capacity.

A number of pathways associated with cellular differentiation emerged, including those involved in cell cycle regulation, loss of pluripotency and AHR and MAPK/ERK signalling pathways. Repair of DNA and S phase checkpoint control were consistently identified throughout chorionic girdle development as important effector networks. Additionally, transcription factors E2 factor (E2F) and E2 factor 2 (E2F2) were identified as top upstream regulators at all time points in the chorionic girdle. The canonical E2Fs (E2F1, E2F2 and E2F3) regulate trophoblast giant cell differentiation through the promotion of the cell endocycle, via regulation of cyclin E and S phase control (Chen et al. 2012, Ouseph et al. 2012). A conserved role for E2F proteins in regulation of cyclins D and E and subsequent G1 cell cycle progression has been observed in human placental cell differentiation (Ohtani et al. 1995). The data generated here suggest that the role for E2F and E2F2 are also conserved in the regulation of the cell cycle in differentiating equine chorionic girdle trophoblasts. Canonical pathway heatmaps indicated that cyclin signalling pathways were upregulated in the chorionic girdle at day 30 of pregnancy, with a subsequent loss of activation at days 31 and 34. Equine trophoblasts

Figure 5 ELF5 mRNA is expressed in chorionic girdle trophoblast cells, correlates with CG/LHB mRNA expression and drives CG/LHB promoter activity. (A) ELF4 and ELF5 mRNA in equine chorionic girdle and control chorion tissues at days 27, 30, 31 and 34 of gestation as shown by RT-PCR with B-actin used as a loading control. Kidney and spleen tissues are used as control. (B) Correlation between ELF5 and CGB mRNA expression in individual day 30–34 equine chorionic girdle and chorion samples. Microarray expression data is normalised to the median in R software and displayed as fold change. (C) Truncated promoter inserts, as shown with annotated potential ELF5 binding sites, were transfected into COS7 cells alone, in the presence of 150ng pCMV-myc-Empty or 150ng pCMV-myc-ELF5. Numbers are relative to Translation Start Site. Promoter activity is expressed as fold change activity over pGL3-basic alone. **P<0.01, ***P<0.001. (n=3, two-way ANOVA) activity is expressed as fold change activity. Over pGL3-basic alone. **P<0.01, ***P<0.001. (n=3, two-way ANOVA).

Figure 6 Activity of the ERK1/2 pathway in the chorionic girdle. (A) Representative western (n=3) for phospho-ERK and total ERK in one timecourse set of chorionic girdle and chorion tissues. Shown are the phosphorylated and total p44 (ERK2) and p42 (ERK1) protein. (B) Densitometry analysis of western blots for phospho/Total ERK (P44/42) n=3. *P<0.05. (C) Multiplex qRT-PCR was used to quantify mRNA expression of SRF and CREB1. *P<0.05, **P<0.01, ***P<0.0001 (compared to day 27 chorionic girdle), ****P<0.001 (compared to chorion at same timepoint) (two-way ANOVA).
Regulation of chorionic girdle development

Allen & Moor 1972

Nevertheless, it is clear that regulation of EGF in human trophoblast, beyond early work that showed a role for metalloproteinases (Vagnoni et al. 1995). In human trophoblast, Rho A mediates EGF-regulated migration (Han et al. 2010). Based on the induction of EGF in pregnant endometrium in mare pregnancy (Allen et al. 2017), it is plausible that this molecular pathway is also important for regulating trophoblast migration in the mare.

Multiple analyses suggested the integrin signalling pathway to be activated increasingly in the chorionic girdle between days 27 and 34 of development. Integrin α2 (ITGA2) expression was a top 20 upregulated gene at day 34 consistent with previous work (Sones et al. 2010). Regulation of binding of collagen and laminin is a well-defined role of ITGA2 and as these are also known to be expressed in mare endometrium (Mansour et al. 2003). Collectively, this is supportive of a role for integrin signalling in invasion of the maternal endometrium around day 36 of pregnancy. Activation of ERK signalling by integrin pathways is thought to play a major role in cell migration, through phosphorylation of myosin chains (Kumar 1998). It is possible that interactions between integrin and ERK signalling is key in co-ordinating the differentiation and migration processes in the chorionic girdle. Whether the trophoblasts migrate to the apices of the girdle folds to differentiate into binucleate eCG-producing cells or they first differentiate and later gain the ability to move towards chemotactic signals is not clear, but this co-ordinated differentiation and cell movement during the development of the chorionic girdle is key and occurs prior to acquisition of invasive potential by trophoblast between days 34 and 38 of pregnancy.

Migration of cancer cells and progression of tumour were identified as some of the top regulator effector networks in the chorionic girdle at all time points. Additionally, the majority of pathways identified in KEGG pathway analysis of gene clusters 1 and 7 were also cancer signalling pathways, with 13 different cancer signalling pathways emerging in total. Trophoblasts have been directly compared to cancer cells in their ability to proliferate, invade the maternal endometrium, establish a blood supply and moderate the maternal immune response (Reeves & James 2017). However, unlike cancer cells, chorionic girdle cells possess an unknown mechanism by which invasion is halted, upon formation of the endometrial cups. Further, chorionic girdle trophoblast senesces upon terminal differentiation, and primary cell cultures have not been maintained much beyond 180 days of culture (Allen & Moor 1972, Thway et al. 2001). Investigating how trophoblast cells regulate their invasion may prove highly informative not only in the field of placentation biology, but also for identification of novel therapeutic targets in invasive cancers.

A number of canonical pathways involved in immune signalling were identified in analysis of the microarray.
data. These included CD28 signalling, cluster of differentiation 40 (CD40) signalling and interleukin 9 (IL-9) signalling, all of which peaked in activation at day 34 of pregnancy. Previous work has shown that equine trophoblast cells are able to resist immune destruction when transplanted across allogenic barriers consistent with an inherent ability to modulate the immune response (de Mestre et al. 2011). IL-9 secreted by human syncytiotrophoblast have been found to play a role in regulation of integrins to promote cellular migration (Sharma et al. 2016). It is therefore possible that a novel role for IL-9 in regulation of endometrial invasion in the equine conceptus through either immunomodulation and/or upregulation of integrin signalling pathways has been identified here. CD40 and CD28 signalling pathways are previously undocumented in placental trophoblast cells of horse, human or mouse origin with further studies required to confirm their activity here. CD40 is commonly found on antigen presenting cells and CD28 on T-helper cells. Uptregulation of these signalling pathways would be expected in the immune cells in the maternal endometrium upon invasion by chorionic girdle trophoblasts, and further investigation of the possible activity and function of these pathways in trophoblast is warranted.

In conclusion, through analysis of microarray data using multiple pathway analysis tools, we have been able to generate an overall impression of the genes and signalling landscape in the chorionic girdle within a 7-day period significantly expanding our understanding of molecular networks in equine trophoblast. Functional assays provided further evidence suggesting roles for ELF5 in biochemical differentiation of trophoblast, Rho A in trophoblast migration and ERK1/2 activity that is likely to mediate FGF7 and EGF signalling during chorionic girdle development. Interrogation of pathway components with in vitro experimentation will further uncover which of these pathways are crucial for the processes of cell movement, differentiation and invasion not only allowing us to gain a more comprehensive understanding of trophoblast biology in horses but possibly informing future work in other species.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-18-0270.

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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