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An ancestral burst of regulatory and protein innovation drives divergent implantation in eutherian mammals.

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ABSTRACT

The origin of placentation in mammals required a symphony of events from protein innovation to the evolution of regulatory networks that could support successful pregnancy from implantation to full gestation. There is variation across mammals in terms of the types of implantation, degree of invasiveness of the trophoblast into the maternal endometrium, and the type of placenta formed. However, there are some signalling networks, e.g. progesterone receptor signalling, that are shared across the clade. We wished to determine what regulates the diverse molecular interactions and morphologies that underpin successful pregnancy in eutheria. Whilst the relationship between miRNAs and pathophysiology of placenta is well established, their role in early implantation and diversity of implantation strategies is not well understood. We identify a cohort of miRNAs that arose coincident with the emergence of placental mammals (mir-127, -185, -188, -28, -324, -331, -340, -378, -423, -433, -505, -542, and -671). We identify 115 genes under positive selection on the stem eutherian lineage and 88 of these are predicted to be regulated by the 13 stem lineage miRNAs. Using two species of mammal that demonstrate the extremes of implantation strategies (invasive and superficial in humans and bovine respectively), we assessed the response of the endometrial epithelium in terms of expression of these miRNAs to early pregnancy molecules. We show that the 13 stem lineage miRNAs are regulated in a species-specific manner. We propose these species-specific regulatory networks contribute to the diversity of pregnancy morphologies observed in eutheria.

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INTRODUCTION

Successful pregnancy in eutheria is contingent, not just on a developmentally competent embryo and appropriate endometrial function, but formation of the transitory organ, the placenta. For eutheria, placenta arises from the outer cells of the embryo (the trophoblast cell lineage) which comes into close proximity with the maternal uterine endometrial tissue during the implantation period to support pregnancy prior to establishing the placenta (Bellomo *et al.*, 1996; Carter and Enders, 2004; Freyer and Renfree, 2009). For successful pregnancy in placental mammals, the regulatory networks that allow the interactions between the temporary organ the placenta and the endometrium likely coevolved (Lynch *et al.*, 2015; Kin *et al.*, 2016).

In eutherian mammals there is wide variation in the type of implantation, the degree of invasiveness of the trophoblast into the maternal endometrium, and the type of placenta formed. The two extremes in terms of implantation strategies are “invasive” (e.g. primates) and “superficial” (e.g. ruminants) (McGowen *et al.*, 2014). The molecular cues during maternal recognition of pregnancy and the morphology of the embryo at the time when this occurs differ between species. In humans for example, chorionic gonadotrophin (hCG) is secreted from the hatched blastocyst that invades into the endometrial epithelium during days 6-8 of pregnancy (Fishel, Edwards and Evans, 1984). In contrast, the bovine conceptus (embryo and associated extraembryonic membranes) is elongated, produces a type 1 interferon (IFNT) (Godkin *et al.*, 1982), and superficial implantation occurs ~day 19 of pregnancy (Figure 1) (Guillomot, 1995; Fair, 2016).

Notwithstanding this diversity in early pregnancy events amongst eutheria, there are conserved signalling pathways that modify endometrial function to facilitate pregnancy success. For example, irrespective of implantation strategy, all eutheria studied so far require the sustained actions of the hormone progesterone (P4) on the endometrium as a prerequisite to establish uterine receptivity to implantation (Spencer and Bazer, 1995). Therefore, regulation of endometrial function in eutheria can be triggered by the same signal (as is the case for progesterone signalling) and/or different molecules resulting in the same outcome (as is the case for maternal recognition of pregnancy signalling).

It has been established that protein coding alterations (such as the birth of new genes and gene families, gene loss, co-option, and selective pressure variation), along with

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innovations in regulatory networks drive the development of novel traits (Wagner, 2015). Whole genome scale analyses have revealed 357 novel protein coding homologous groups that emerged on the eutherian stem lineage (Dunwell, Paps and Holland, 2017). In addition, an analysis of 2218 genes associated with endometrial expression on the stem eutherian lineage, identified 835 genes co-opted into endometrial function on the eutherian stem lineage (Lynch *et al.*, 2015). And whilst the cooperative interaction between *FOXO1A, HOXA11, FOXO1A* and *CEBPB* evolved on the stem eutherian lineage - they facilitate decidualization of the endometrium (a process required for implantation) in only a select set of eutheria, e.g. humans and mice (Kin *et al.*, 2016). There are a number of cases of selective pressure variation in protein coding genes that have had a direct role in endometrial function. The primate-specific hormone chorionic gonadotropin, the maternal recognition of pregnancy signal in humans is comprised of an alpha (CGα) and a beta (CGβ) subunit (Morgan and Canfield, 1971) with the CGβ subunit originating from a duplication of the luteinizing hormone subunit (LHβ) in the ancestor of anthropoid primates (Maston and Ruvolo, 2002). Members of the galectin family of proteins involved in immune modification are expressed at the maternal-fetal interface were also found to be under positive selection in primates, including 5 galectins specifically expressed in the placenta (Than *et al.*, 2009) as well as cadherins, a family of cell surface adhesion proteins (Summers and Crespi, 2005).

Significant miRNA family expansions have been found to correlate with major transitions in animal evolution, e.g. the miRNA repertoire expanded on the *Bilaterian* stem lineage (Hertel *et al.*, 2006; Prochnik, Rokhsar and Aboobaker, 2007; Grimson *et al.*, 2008). The role of miRNAs in regulation of tissue function is clear from studies of healthy placental development and successful pregnancy. For example, miR-675 is exclusively expressed in the placenta, while knockdown results in placental overgrowth (Keniry *et al.*, 2012). Temporal expression of members of the miR-17~92 cluster in trophoblast cells alters differentiation (Kumar *et al.*, 2013). Aberrant miRNA expression plays a role in pathophysiology of pregnancy. Elevated miR-210, miR-1233 and miR-574 are associated with preeclampsia (Munaut *et al.*, 2016), and miR-133a with recurrent miscarriage (Santamaria and Taylor, 2014).

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Physiological factors can also alter miRNA expression. Oestrogen expression has been found to alter the expression of multiple miRNAs, including miR-29 (Zhang *et al.*, 2012); miR-26a and miR-181a (Maillot *et al.*, 2009); and miR-879 (Wetzel-Strong *et al.*, 2016) while miR-21, a miRNA highly expressed in human tumours and cancer cell lines, is induced by the binding of Type I Interferon induced STAT3 to a promoter region upstream of mir-21 (Yang *et al.*, 2015). It is clear that miRNAs may be stimulated by multiple processes including steroid hormones and Interferons - but the species specificity of this regulation is still unknown.

Whilst the relationship between miRNAs and pathophysiology of placenta have been well established, the more fundamental question of their role in early implantation and diversity of implantation strategies persists. We are interested in determining what regulates the diverse molecular interactions and morphologies that underpin successful pregnancy in eutheria. Specifically we set out to: (1) identify miRNAs families that arose when eutheria arose and were retained in all extant eutheria sampled, (2) to estimate the set of positively selected SGOs on the eutherian lineage and whether they are preferentially targeted by these miRNAs, and, (3) understand how these miRNAs are regulated by key molecules important for early pregnancy success in two species with different implantation strategies. We identify a cohort of miRNAs that arose co-ordinate with the emergence of placental mammals. We find they are regulated in a species-specific manner in the endometrial epithelium of two diverse eutheria and preferentially bind to protein coding genes that underwent positive selection in the stem eutherian lineage. We propose these species-specific regulatory networks contribute to the diversity of pregnancy morphologies observed in eutheria (Figure 1).

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MATERIALS AND METHODS

Identification of miRNAs that emerged on the eutherian stem lineage

Using the standard mammal phylogeny (Morgan *et al.*, 2013; Tarver *et al.*, 2016) and the comprehensive set of microRNAs (miRNAs) in MirGeneDB we identified 112 miRNA families that emerged on either the eutherian or therian stem lineage (Fromm *et al.*, 2020).

The species sampled had representation across the following vertebrate clades: *Fish, Amphibia, Reptilia, Aves, Monotremata, Metatheria and Eutheria* (as a positive control) (Table 1). For each miRNA, the stem-loop sequence (i.e. ~100nt) was used as the query sequence. Homology searches were performed in BLASTn (version 2.6.0+) (Altschul *et al.*, 1990) with e^{-10}. The phylogenetic distribution of the 112 miRNA families was determined from the patterns of presence and absence on the current mammal species phylogeny (Morgan *et al.*, 2013; Tarver *et al.*, 2016) using the parsimony-based approach implemented in TNT (Giribet, 2005).

Computational prediction of the targets of mammal stem lineage miRNAs

TargetScan (Agarwal *et al.*, 2015) was used to predict human targets for the miRNAs that were unique to, and phylogenetically conserved across, the eutheria (hereafter referred to as the "13 stem lineage miRNAs") (Supplementary Table 1). TargetScan precomputed UTR alignments using Multiz (Blanchette *et al.*, 2004) were used for the target site predictions (Agarwal *et al.*, 2015). To determine if the predicted target sites were conserved we assessed sequence conservation across a diverse set of eutherian species, i.e. Human, Cow, Mouse and Elephant. Targets conserved across all four species and with the strongest binding affinity were prioritised for further analysis, i.e. 8mer-A1, 7mer-m8 and 7mer-A1 complementary binding to the seed region (Bartel, 2009). The protein coding target genes were analysed for functional enrichment using PANTHER v.14 (Mi *et al.*, 2019) and for enrichment in pathways using the Reactome Pathway Database (Fabregat *et al.*, 2016).

Single Gene Ortholog Annotation, Alignment, and Filtering for Selective Pressure Analysis

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We chose a total of ten eutheria that demonstrate the greatest range of diversity in placental morphology, plus four outgroup species one from each of the following groups: *Monotremata, Marsupiala, Aves* and *Teleosti* (Table 2). Annotated gene families were taken from Ensembl 90 (accessed 28th November 2017) (Yates *et al.*, 2016). A total of 7,614 homologous gene families were present in all 14 species (10 placental mammals and 4 outgroups), this included paralogs. From these 7,614 homologous gene families, we identified 1,437 gene families that are in single copy in the eutheria - i.e. single gene orthologs (SGOs), had ≥7 species (Anisimova and Yang, 2007) and contained at least one suitable (non-eutherian) outgroup. Multiple sequence alignments (MSAs) were generated using MUSCLE v3.8.1551 (Edgar, 2004) and MAFFT v7.310 (Katoh *et al.*, 2002a) default settings and the output were compared using the 'metal_compare' function in VESPA (Thompson *et al.*, 2001; Blackburne and Whelan, 2011; Webb, Walsh and O'Connell, 2016). The optimal MSA for each SGO was used for selective pressure analysis using codeml from the PAML package (Yang, 2007) wrapped in the VESPA (Webb, Walsh and O'Connell, 2016) pipeline. The resolved mammal phylogeny (Morgan *et al.*, 2013; Tarver *et al.*, 2016) was appropriately pruned for each of the 1,437 SGOs using VESPA so that only those species in the alignment were in the corresponding tree for a given gene family (Webb, Walsh and O'Connell, 2016). Aligned amino acids were used in combination with the corresponding nucleotide data to generate aligned nucleotide files (Webb, Walsh and O'Connell, 2016).

Analysis of Selective Pressure Variation

VESPA (Webb, Walsh and O'Connell, 2016) automated the process of applying site-specific and lineage-site specific codeml models from the PAML package (Yang, 2007) across the set of 1,437 SGOs, where the eutherian stem lineage was treated as foreground. We identified those putative positively selected residues on the eutherian stem lineage that were subsequently fixed in all extant eutheria. SGOs with evidence of positive selection where the residue was fixed in all extant eutheria were analysed for functional enrichment using GO Slim Biological Process terms and PANTHERv.14 (Mi *et al.*, 2019). STRINGv.11 database of interactions (Szklarczyk *et al.*, 2019) was used to determine if these genes interacted significantly more than expected by chance.

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Assessing if the 13 stem lineage miRNAs have significant levels of targeting to the positively selected SGOs

The 13 stem lineage miRNAs were assessed for predicted targets in the SGOs that displayed evidence of positive selection on the eutherian stem lineage. As there were 115 SGOs under positive selection - we randomly sampled 100 sets of 115 genes from the human genome (ensuring that our positively selected cohort were not amongst them), and we predicted targets for the 13 stem lineage miRNAs across all 100 random samples. We tested our null hypothesis that there is no difference in levels of target sites predicted for the positively selected cohort versus the randomly sampled genes using a two-sample t-test with unequal variance, determined using Levene's test of variance of populations (Levene, 1960).

MODIFICATION OF STEM LINEAGE miRNAS IN IN VITRO MODELS OF EARLY PREGNANCY.

Unless stated, all chemicals and consumables were sourced from Sigma-Aldrich, UK.

Bovine Epithelial Cell Culture

The effect of P4 was assessed using cells isolated from tracts in the early luteal phase. The effect of conceptus-derived proteins were assessed in cells isolated from late luteal phase tracts to better mimic events in early pregnancy *in vivo*. Tracts were staged (Ireland, Murphee and Coulson, 1980) and cells isolated and cultured as previously described (Tinning *et al.*, 2020). Briefly, the ipsilateral horns were dissected by inserting sterile curved scissors into the uterine lumen and endometrium washed once with PBS supplemented with 1% GSP (Thermo Fisher Scientific, USA). The endometrium was dissected from the myometrium in sheets and washed in HBSS (1% GSP) and chopped into 3-5mm fragments and digested for 1 hr at 37°C in 40mL HBSS (10% BSA, 20mg collagenase II, 4% DNase I, and 100X Trypsin solution). Digested tissue was passed through a 40µM cell strainer to isolate epithelial cells, and a 70µM strainer to isolate stromal cells. Cells were maintained in T75 flasks, in RPMI (10% FBS Gold, 1% ABAM) in a 37°C incubator at 5% CO_2 for 6 days, passaging every 3 days, until the cells reached 70% confluency. Cultured epithelial and stromal cells (n=3 biological replicates) were counted using Trypan Blue exclusion dye. Epithelial cells were diluted to 100,000 cells/mL and plated at 2mL/well in 6 well plates prior to treatment.

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Cells isolated from early luteal phase endometria were treated for 24 hrs with one of the following: 1) Control, 2) Vehicle (EtOH), 3) 0.1µg/mL P4, 4) 1.0µg/mL P4, or 5) 10.0µg/mL P4. Cells isolated from late luteal phase endometria were treated with either: 1) control, 2) vehicle control (PBS), 3) 1000ng/mL recombinant ovine IFNT, 4) 1000ng/mL recombinant bovine CAPG (bCAPG), or 5) 1000ng/mL recombinant bovine PDI (bPDI). After 24 hr of treatment cells were trypsinized, pelleted, and lysed using the Invitrogen *mir*Vana miRNA extraction lysis buffer. Lysed cells were transferred to a sterile labelled tube, snap-frozen in liquid nitrogen, and stored at -80°C.

Human endometrial epithelial cell culture

Ishikawa cells (ECACC: #99040201) were maintained in Gibco Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Thermo Fisher Scientific, USA) with the addition of 10% (v/v) Gibco One-shot EV-depleted FBS (Thermo Fisher Scientific, USA) (charcoal stripped using 1g dextran-coated charcoal) and GSP (1%). Cells were incubated at 37°C and 5% CO_2 in a T75 flask until they reached 70% confluency, counted using Trypan Blue exclusion dye, diluted to 100,000 cells/mL and plated at 2mL cells/well in a 6 well plate. Cells received the following treatments (n=3 biological replicates): 1) control, 2) vehicle control (20µl PBS), 3) 1000ng/mL bCAPG or 4) 1000ng/mL bPDI for 24 hr. Cells were trypsinized, pelletted, and lysed using the Invitrogen *mir*Vana miRNA extraction lysis buffer (Invitrogen), transferred to a sterile labelled tube, snap-frozen in liquid nitrogen and stored at -80°C. For those cells treated with hCG treatment, they were pre-treated with P4 (10µg/ml) for 24 hr, media replenished and treated with one of the following for 24 hr (n=3): 1) control, 2) vehicle control (10µl ethanol, 10µl PBS), 3) P4 only (10µg/ml), or 4) P4 (10µg/ml) and hCG (10µg/ml) (Ray Biotech, USA). After 24 hr cells were collected as described above.

RNA Extraction, cDNA conversion and miRNA expression analysis

Total RNA from all samples (except the hCG treated cells which were extracted using the miRNeasy Mini Kit (Qiagen, UK)) was extracted as per manufacturers' instructions using an acid phenol chloroform extraction method. Following wash steps RNA was eluted from the column into a fresh collection tube using 50µl of nuclease-free water. Eluted RNA was DNase treated using the Invitrogen DNA-free kit, adding 5µl 10X DNase I buffer along with

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1µl DNase I and incubated at 37°C for 30 min. The reaction was stopped using 5µl DNase Inactivation Reagent. Samples were mixed by pipetting and incubated at room temperature for 2 min, and centrifuged at 10,000 g for 15 min. The aqueous phase was collected and RNA content immediately quantified using the NanoDrop N1000 (Thermo Fisher Scientific, USA). Reverse transcription was performed using the miRCURY LNA reverse transcription kit (Qiagen, UK), according to the manufacturer's instructions. RNA was diluted to 5ng/µl using sterile DNase/RNase free water. Two µl of diluted RNA was added to 2µl of 5x miRCURY Reaction Buffer, 1µl of 10x miRCURY Enzyme Mix, and 5µl DNase/RNase free water. Samples were incubated at 42°C for 60 minutes and the reaction was inactivated by incubating at 95°C for 5 minutes. Quantification of miRNAs was performed using miRCURY LNA miRNA Custom PCR Panels, along with 2 normalisation genes and 2 spike-ins (Qiagen, UK; configuration #YCA21533). Cycling conditions were as follows using a Roche LightCycler (UK): 95°C for 2 min, followed by 45 cycles (95°C for 10 sec, 56°C for 60 sec). A melting curve was included (95°C for 1 min and 60°C for 30 sec). Delta Ct values were obtained using 5S rRNA and U6 snRNA normalisation genes. Paired 2-tailed t-tests were performed on dCt values in GraphPad PRISM, comparing vehicle control to treatment samples, where miRNAs were determined to be differentially expressed when $p \leq 0.05$.

Reactome Pathway Analysis of the Targets of Differentially Expressed miRNAs

Targets of all of the 13 stem lineage miRNAs determined to be differentially expressed in *in-vitro* models of early pregnancy in human and bovine samples (Figure 1: $p \leq 0.05$) were converted from Ensembl transcript identifiers to gene names using Ensembl BioMart (Zerbino *et al.*, 2017). Reactome pathways were constructed from these gene names using the Reactome Pathway Database (Jassal *et al.*, 2019) for pooled targets of all miRNAs differentially expressed in a treatment group (e.g. all significantly differentially expressed miRNAs in human Ishikawa cells treated with 1000ng/µl bPDI).

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RESULTS

13 stem lineage miRNAs arose at the origin of therian and eutherian mammals and were never subsequently lost.

In total, we identified 112 miRNA families as originating on the therian or eutherian stem lineage from MirGeneDB (Fromm *et al.*, 2020). Six miRNA families emerged on the Therian stem lineage (mir-340, mir-483, mir-671, mir-675, mir-1251, and mir-3613), of which only mir-340 is phylogenetically conserved in Theria and Eutheria. In contrast, mir-671 is present in all species sampled with the exception of Opossum. Considering mir-671 is present in tasmanian devil and it is also present in all Eutheria sampled, we considered mir-671 as a 'therian stem lineage miRNA'. A total of 106 miRNAs emerged at the origin of *Eutheria* and 11 of these remained conserved in all extant *Eutheria* sampled (Figure 2). Publicly available expression sets of miRNAomes (Fromm *et al.*, 2020) suggest that these miRNA families may be expressed in placental tissue.

Targets were identified in the human genome for these 13 stem lineage miRNAs using TargetScan (Agarwal *et al.*, 2015). We found 22,911/29,264 annotated 3'UTRs with predicted targets (see materials and methods for filters). However, the number of predicted binding sites for each miRNA varied, *e.g.* miR-127 was predicted to target 520 transcripts, whilst miR-185 had 20,754 predicted target transcripts (Figure 3a). Predicted functions of the targets of these 13 miRNAs include: reproductive functions (55 target transcripts), metabolic process (1476 target transcripts), Biological regulation (1269 target transcripts) (Figure 3b). Included in the list of targets for the 13 stem lineage miRNAs are the Syncytins, endogenous retroviruses that promote placental formation (Esnault *et al.*, 2013). Specifically, miR-185-3p, miR-188-3p, miR-423-5p and miR-433-3p are predicted to target the syncytins with at least 7-mer binding and miR-423-5p has two predicted target sites on *syncytin-1*, with one site overlapping that of miR-185-3p, indicating a dynamic/competitive binding action.

Evidence of positive selection on the stem eutherian lineage.

From a total of 1,437 SGOs, MAFFT (Katoh *et al.*, 2002b) was determined to be the best fit multiple alignment method for 87% of SGOs, and MUSCLE (Edgar, 2004) for 13% SGOs. We identified signatures of positive selection on the stem eutherian lineage in 237 SGOs, 115 of which contained amino acid residues that were subsequently unaltered in all daughter

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lineages. The functions of these positively selected SGOs are predominantly cellular processes, metabolic processes, and biological regulation (Figure 4a). The 115 SGOs that underwent positive selection on the stem eutherian lineage were found to be functionally enriched for telomere activity and were implicated in pathways related to chromosomal maintenance, telomere activity, p53 signalling, cell cycle and the inflammatory immune response, activities which have been associated with the formation of the placenta (Hauguel-de Mouzon and Guerre-Millo, 2006; Woods, Perez-Garcia and Hemberger, 2018; Gal *et al.*, 2019). Furthermore, 84 of the 115 PSG families are predicted to be targeted by the 13 stem lineage miRNAs (Figure 5). Of these SGOs, 21 are significantly more likely to interact with one another ($p<0.05$) in comparison to any other gene in the genome (Figure 4b). A summary of the major functional annotations enriched in these 84 PSGs predicted to be regulated by the 13 miRNAs identifies these genes as involved in cell cycle, DNA damage and DNA metabolic processes, along with genes involved in regulation of hair cycle and hair cycle – a uniquely mammal characteristic (Figure 6). Among these are three functionally related genes: WD Repeat Containing Antisense to TP53 (WRAP53), telomerase reverse transcriptase (TERT), and, ACD shelterin complex subunit and telomerase recruitment factor (ACD). These genes are all involved in telomerase function, WRAP53 interacts with TERT, and ACD is one of six proteins that function in the telosome/shelterin telomeric complex maintaining telomere length and protecting the telomere ends (Xin, Liu and Songyang, 2008). The WRAP53 protein has dual function and also serves as an antisense transcript of tumor protein p53, positively regulating p53 expression by targeting the 5'UTR of p53 (Mahmoudi *et al.*, 2009). WRAP53 contains a WD40 repeat domain which assists in the localisation of Cajal bodies, small nuclear bodies involved in telomere maintenance (Henriksson and Farnebo, 2015) and there are 6 positively selected sites within the WD40 repeat. Within TERT, 5 of the positively selected residues are within the RNA-interacting domain 1 and 2 within the C-terminal extension (Wyatt, West and Beattie, 2010). ACD recruits the POT1 protein to protect chromosome ends (Xu *et al.* 2019) - 8 of the positively selected residues are within the POT1 interacting domain of ACD (Xu *et al.*, 2019).

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The 13 stem lineage miRNAs preferentially target genes that underwent positive selection on the stem Eutherian lineage

Out of 115 SGOs with evidence of positive selection on the ancestral eutherian lineage, 84 were found to be significantly enriched for binding sites for the 13 stem lineage miRNAs (p=1.35618e-11), with a mean=6.66 binding sites per transcript (median=4.0). We wished to determine if this number of binding sites in this subset of positively selected genes was significantly different than one would expect by random chance given a similar sample of genes in the genome. The binding sites per transcript for the 13 stem lineage miRNAs in the 100 random sample sets of genes is significantly lower, mean=1.636 (median=1.0). This suggests that the 13 stem lineage miRNAs preferentially target positively selected SGOs (Figure 5).

Species-specific regulation of the 13 stem lineage miRNAs by key early pregnancy molecules in endometrial epithelial cells of species with different implantation strategies.

We have identified a response in expression of 11/13 of the stem lineage miRNAs in the endometrial epithelial cells of bovine and/or human (Figure 1). Treatment of bovine endometrial epithelial cells with 10µg/mL of P4 during the early luteal phase resulted in increased expression of miR-505-5p (Figure 7). In contrast, addition of the pregnancy recognition signals (IFNT in bovine: hCG in human) to receptive endometrial epithelial cells did not alter expression of the 13 stem lineage miRNAs (p>0.05: see Supplementary Figure 1 for IFNT data).

Two recently identified proteins that are produced by the conceptus during pregnancy recognition are CAPG and PDI, they are proposed to modify the endometrial to facilitate the implantation process (Forde *et al.*, 2011; Tinning *et al.*, 2020). Addition of recombinant bovine forms of bCAPG and bPDI proteins to bovine or human endometrial epithelial cells changed expression of selected miRNAs in a species-specific manner (Figure 8 and 9 respectively). Treatment of bovine cells with recombinant bCAPG resulted in increased expression of miR-331-3p, miR-324-5p and miR-505-5p in endometrial epithelial cells (p<0.05). Expression of miR-127-3p, miR-151a-3p (a paralog of mir-28 originating on the Eutherian stem lineage), and miR-188-5p showed a significant decrease in expression in human endometrial epithelial cells treated with 1000ng/µl bCAPG compared to vehicle control (p<0.05: Figure 8).

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Treatment with recombinant bPDI decreased expression of miR-185-5p in bovine epithelial cells ($P<0.05$). Expression of miR-151a-5p, miR-185-5p, miR-378a-3p and miR-532-5p (a paralogue of mir-188 originating on the Eutherian stem lineage), showed a significant decrease in expression in human Ishikawa immortalised endometrial epithelial cells treated with 1000ng/µl bPDI compared to vehicle control (Figure 9).

DISCUSSION

In order for placental mammals to have arisen, it was necessary for precise molecular choreography between the embryo and the endometrium to have coevolved. These regulatory interactions are key for implantation success and are arguably the single most critical point in the process of *in utero* development leading to live birth - a hallmark of placental mammals. Implantation within the eutherian clade is not fixed, rather there is a diversity of implantation strategies known. We tested the hypothesis that successful implantation in placental mammals is facilitated by the emergence of regulatory and protein coding innovation at their origin. We identify a set of 13 miRNA families that originated on the stem mammal lineage, and that are regulated by molecules known to facilitate implantation. In addition, our comparison of bovine and human endometrial epithelial cell types demonstrates that the expression or repression of these 13 miRNAs are species specific in nature (Figure 1). We find evidence for positive selection (indicative of protein functional shift) on the ancestral eutherian node in 115 single gene orthologous families. From our target site prediction, the 13 stem lineage miRNAs preferentially regulate these 115 SGOs. We have identified key regulatory networks and signatures of adaptive evolution that are necessary for the establishment of implantation in two diverse Eutheria (bovine and human), and as such were likely to be key to mammal evolution.

Expression of some of the 13 stem lineage miRNAs has been demonstrated for normal placental (e.g. mir-433: (Ito *et al.*, 2015), mir-28: (Farrokhnia *et al.*, 2014), mir-378: (Luo *et al.*, 2012)), and endometrial (e.g. mir-505 (Morales-Prieto *et al.*, 2012)and mir-542 (Tochigi *et al.*, 2017)) functions. Others have been implicated in pathophysiological pregnancies, e.g. preeclampsia: mir-185, mir-188, mir-423 and mir-542: (Fu *et al.*, 2013; Harapan and Andalas, 2015; Hosseini *et al.*, 2018); placentomegaly: mir-127 (Ito *et al.*, 2015); LGA pregnancies: mir-324 (Rahman *et al.*, 2018); placenta from intra-amniotic infection: mir-331 (do Imperio *et al.*, 2018), and preterm birth: mir-505 (Fallen *et al.*, 2018)).

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Collectively these 13 miRNAs have been implicated in critical roles in placental mammal pregnancies. Here were focus on how these miRNAs may regulate the diversity of implantation strategies observed in human and bovine during the implantation phase of pregnancy.

Novel protein coding elements have emerged in mammals and have been shown to have critical roles in successful implantation, e.g. ERVs (Emera and Wagner, 2012; Spencer and Palmarini, 2012; Chuong, 2013). Large-scale comparative genomic studies have identified 357 novel gene families (Dunwell, Paps and Holland, 2017) and adaptation of existing gene families to new functions on the ancestral eutherian node (Gibbs, Roelants and O'Bryan, 2008; Kosiol *et al.*, 2008; Cardoso-Moreira *et al.*, 2019). As positive selection has been shown to be synonymous with protein functional shift (Lan, Wang and Zeng, 2013) this suggests a significant level of alteration of these functions at the origin of eutheria. However, a synergy between regulatory and protein coding innovations are necessary to drive substantial phenotypic novelty (Lynch *et al.*, 2011; Wagner, 2015; Kin *et al.*, 2016). By focussing on miRNA regulatory networks, which have been shown to correlate significantly with phenotypic diversity (Hertel *et al.*, 2006; Prochnik, Rokhsar and Aboobaker, 2006; Grimson *et al.*, 2008), we establish a statistically significant relationship between the 13 miRNAs that emerged on the ancestral mammal lineage and genes under positive selection on the ancestral eutherian lineage. The predicted targets of these miRNAs included 130 gene families that emerged on the eutherian stem lineage (Dunwell, Paps and Holland, 2017). The targets of these miRNAs were implicated in pathways associated with INPP5E regulation, Neurophilin interactions and VEGF interactions with its receptor (VEGR), each implicated in angiogenesis. TGF-β signalling and p53 regulation were also among the associated pathways, which are each implicated in cell proliferation. Amongst the predicted targets were 15,379 genes that evolved endometrial expression on the stem eutherian lineage that are hypothesized to have assisted in the remodelling of the uterine landscape during the evolution of the placenta (Lynch *et al.*, 2015). We propose that this preferential targeting and protein functional shift were essential to the establishment of mammalian implantation, and that subsequent diversification of this network facilitated the range of implantation strategies observed today.

We evaluated the regulation of the 13 stem lineage miRNAs in the endometrial epithelia from humans and bovine as the epithelia is the first cell that the trophoblast of the

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embryo encounters, and the bilateral communication between these tissues is well documented (Schlafke and Enders, 1975; Kim and Kim, 2017; Guillomot, 2019). Human and bovine last shared a common ancestor some ~92 million years ago, representing ~184 million years of independent evolution (Liu *et al.*, 2006; Donoghue and Benton, 2007). They represent two distinct implantation strategies for mammals, with humans being invasive and bovine being superficial in nature. Comparison of human and bovine allows us to perform a direct comparison in terms of response by the miRNAs to the same (e.g. PDI, CAPG) or equivalent (e.g. hCG, IFNT) molecular stimuli. Our data has shown that the response is, for the most part, species-specific, i.e. treatment of these 2 species with the same stimulus elicits species-specific miRNA responses (Figure 1). These results are conducive with the diverse morphological implantation strategies known for human and bovine (Bauersachs *et al.*, 2006; McGowen *et al.*, 2014; Kim and Kim, 2017). None of the stem lineage miRNAs are regulated by the species-specific pregnancy recognition signals, but they are modified by proteins that are highly conserved amongst placental mammals. Combined, this suggests that the regulatory networks leading to implantation occurred concordantly with placental mammal emergence, but subsequently evolved species-specificity for pregnancy recognition signalling.

In conclusion, using human and bovine *in vitro* models of early pregnancy events, the expression of 11 miRNAs were found to be significantly altered in response key implantation-related molecules. The overrepresentation of positively selected genes in the target set for these 13 miRNAs indicates a role in regulation of novel function that emerged on the Eutherian lineage. We propose that the preferential interactions we observe established the ancestral regulatory network that facilitated the evolution of placental mammals and the subsequent diversity of implantation strategies.

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CONTRIBUTIONS

NF and MJOC conceived of the study and designed the experiments. AST, BC, VO and MJOC carried out all evolutionary analyses and DW carried out statistical tests. NF, AST, HT, WS, ALP, and RAS undertook all molecular analyses. NF and MJOC drafted the manuscript, all authors contributed to revising, reading, and critiquing the manuscript.

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FIGURES

Figure 1:

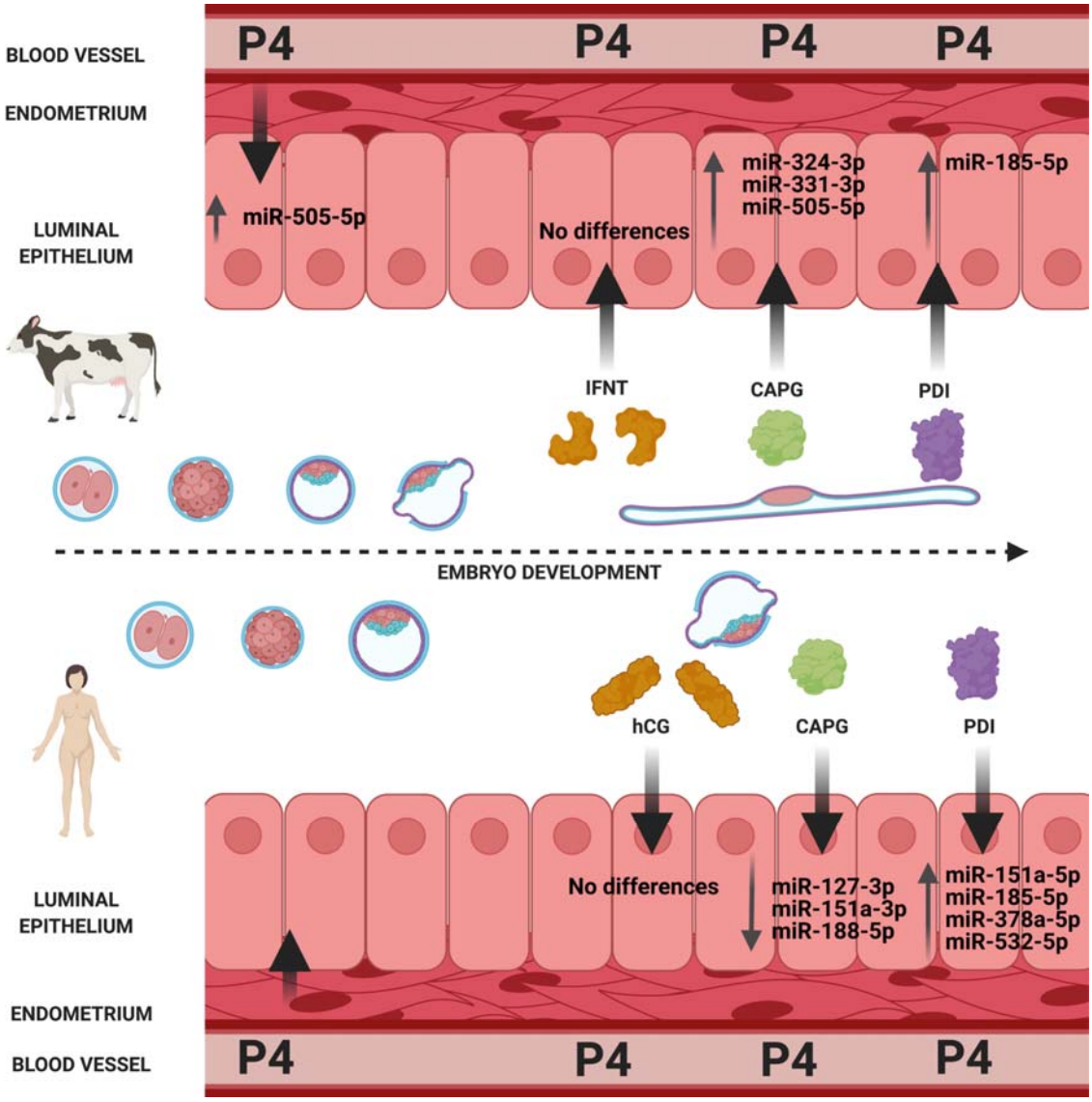

Figure 1. Regulation of 13 stem lineage miRNAs by early pregnancy markers. *The endometrial epithelium of bovine (superficial implantation strategy) (top panel) or human (invasive implantation strategy) (bottom panel) are regulated by molecules important for endometrial function in early pregnancy in eutheria (P4: progesterone; hCG: human chorionic gonadotrophin; Interferon Tau: IFNT; Macrophage capping protein: CAPG, and protein disulfide isomerase: PDI).*

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Figure 2:

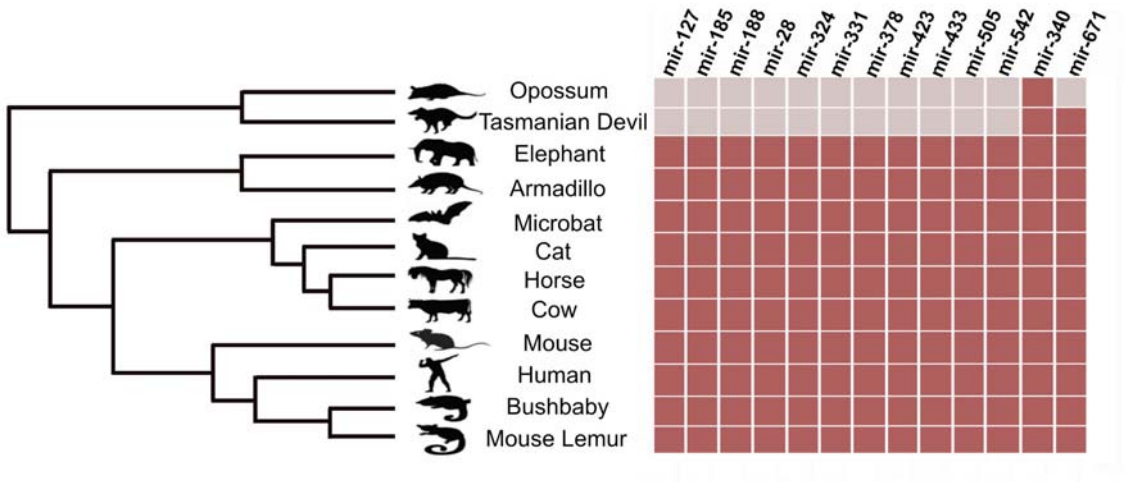

Figure 2. Phylogenetic distribution of miRNA families specific to therian and eutherian mammals. *The current mammal species phylogeny is shown to display the species we sampled in our analysis. The corresponding matrix shows the presence (dark red) or absence (pale grey) of miRNA families across the species sampled.*

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Figure 3:

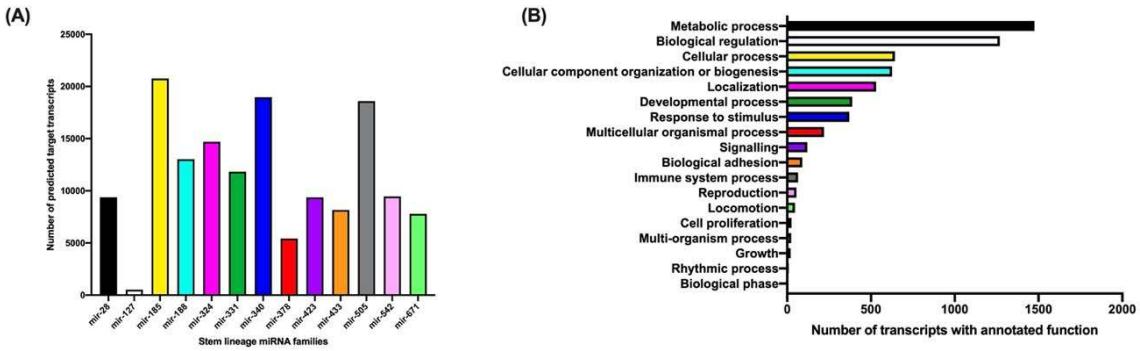

Figure 3. Number of predicted targets for each of the 13 stem lineage miRNA family and the functional annotation of the target genes using Panther DB. (A) *Targets were predicted for each of the 13 stem lineage miRNAs using TargetScan70 (Agarwal et al., 2015). TargetScan output was filtered for targets with8mer-A1, 7mer-m8 and 7mer-A1 complementary binding to the seed region.* ***(B)*** *Functional annotation of predicted targets of the 13 stem lineage miRNAs. Filtered stem lineage miRNA target transcripts were analysed for functional enrichment using PANTHERv.14 (Muruganujan et al., 2018), where PANTHERv.14 found functional annotations to be significantly enriched when p≤0.05.*

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Figure 4:

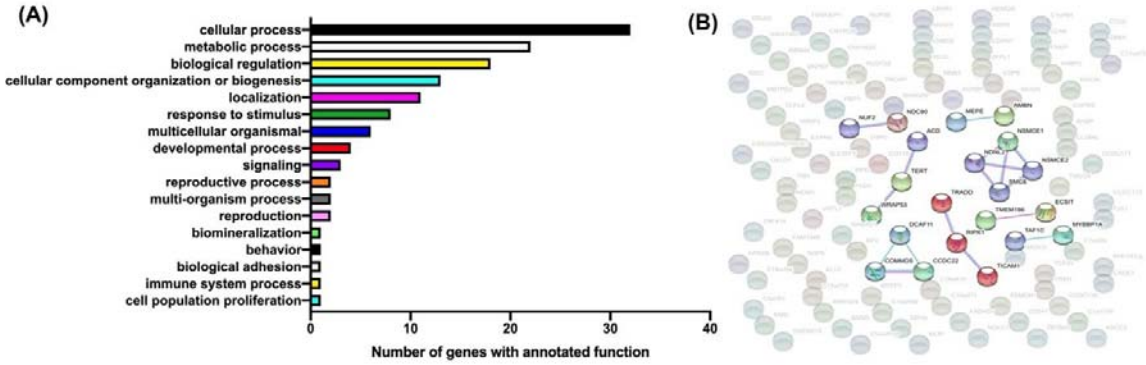

Figure 4. Broad functions of the genes with signatures of positive selection and sample of their interactions. (A) *Functional annotation using Gene Ontology Biological Process terms of 115 SGOs that underwent positive selection on the stem eutherian lineage and where the amino acid substitution was fixed on all extant Eutheria tested. The absolute number (out of 115) of positively selected genes in a given category are shown in the X axis and the functional annotations on the Y-axis.* ***(B)*** *String interaction network of the same set of 115 genes. Network has 106 total nodes and 8 edges (expected edges =4). Background node, with no high confidence interactions from experimental and database sources are translucent. Nodes with high confidence interactions from experimentally determined (pink lines) or curated database (blue lines) sources are depicted in colour. The network was found to be significantly enriched for gene-gene interactions (p=0.0468). Average node degree is 0.151, with an average local clustering coefficient of 0.104. Minimum interaction score is 0.700.*

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Figure 5:

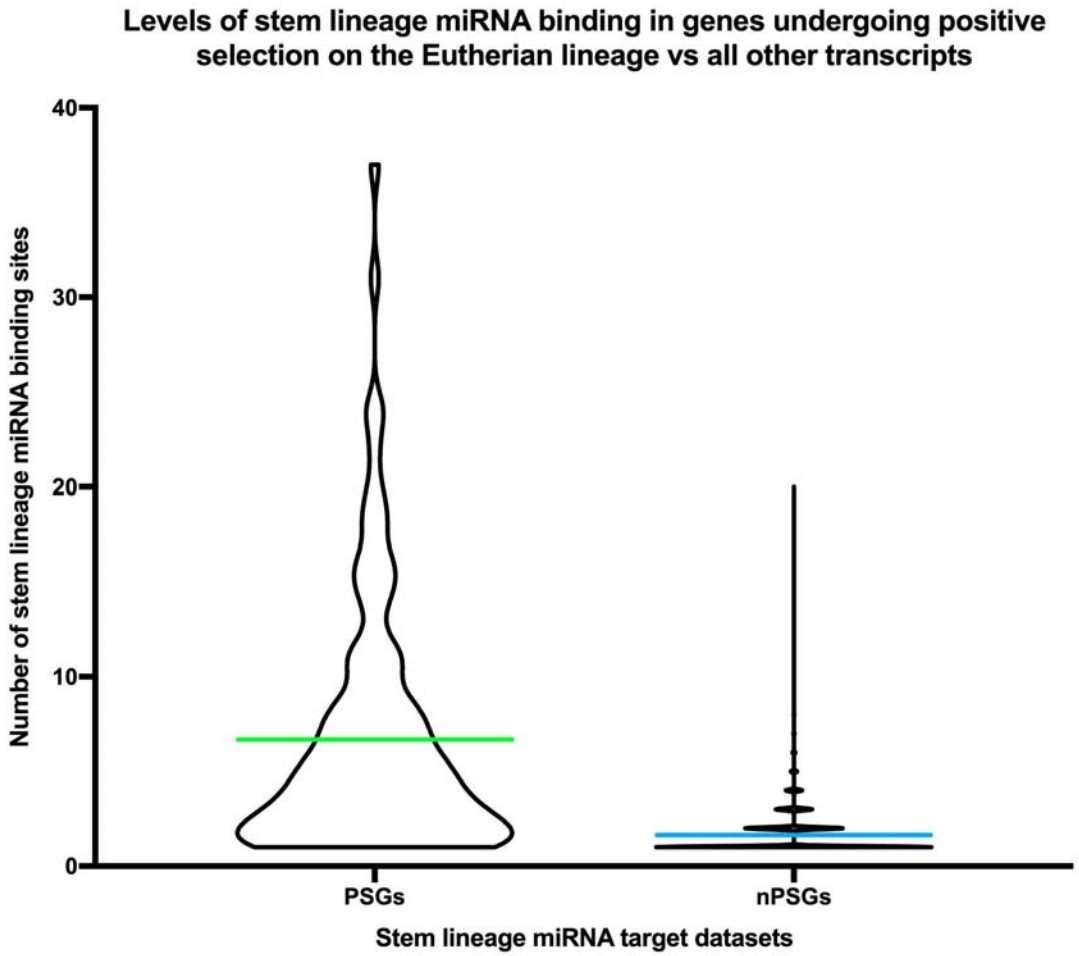

Figure 5. Violin plot showing the number of stem lineage binding sites per transcript in genes that underwent positive selection on the stem Eutherian lineage (PSGs) compared to genes that did not undergo positive selection on the stem Eutherian lineage (nPSG). *For each of the 84 gene families that underwent positive selection on the stem eutherian lineage, the mean number of stem lineage miRNA binding sites (depicted in green) was determined for each transcript of the human gene orthologue. This was compared to the mean number of binding sites for each predicted human target transcript that did not undergo positive selection on the stem eutherian lineage (depicted in blue). The mean number of binding sites was determined to be significantly different between the two datasets when p≤0.05, determined using a two-sample t-test with unequal variance.*

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Figure 6:

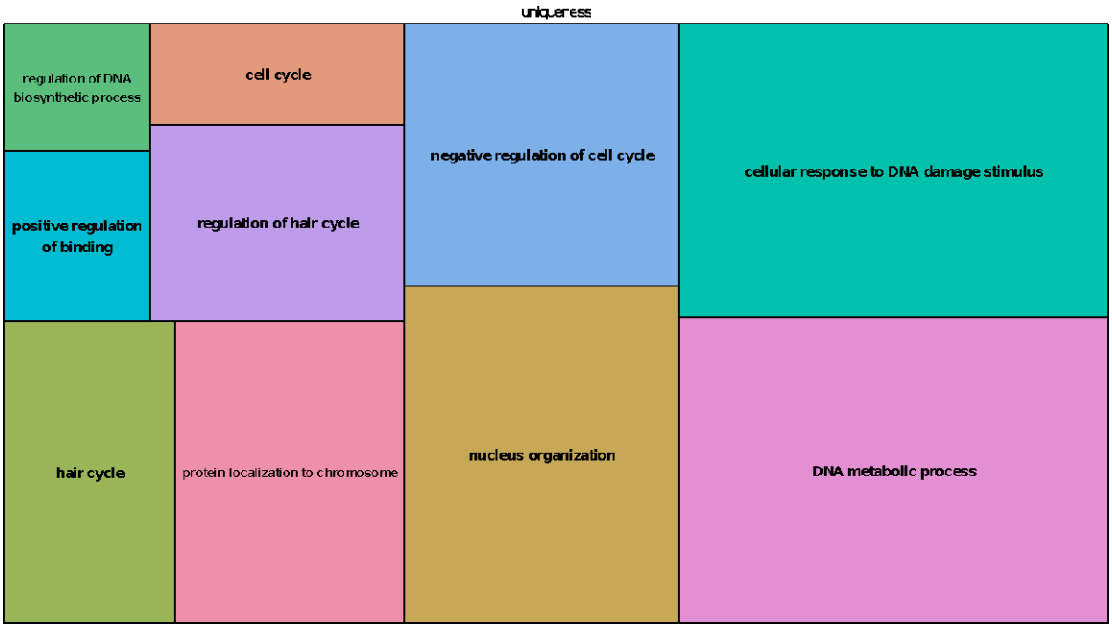

Figure 6: A standard TreeMap from REVIGO displaying the GO biological process terms present in the 84 PSGs that were predicted targets of the 13 stem lineage miRNAs. *Rectangle size represents semantic uniqueness of GO term, defined by REVIGO as the negative of average similarity to all other terms present in human.*

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Figure 7:

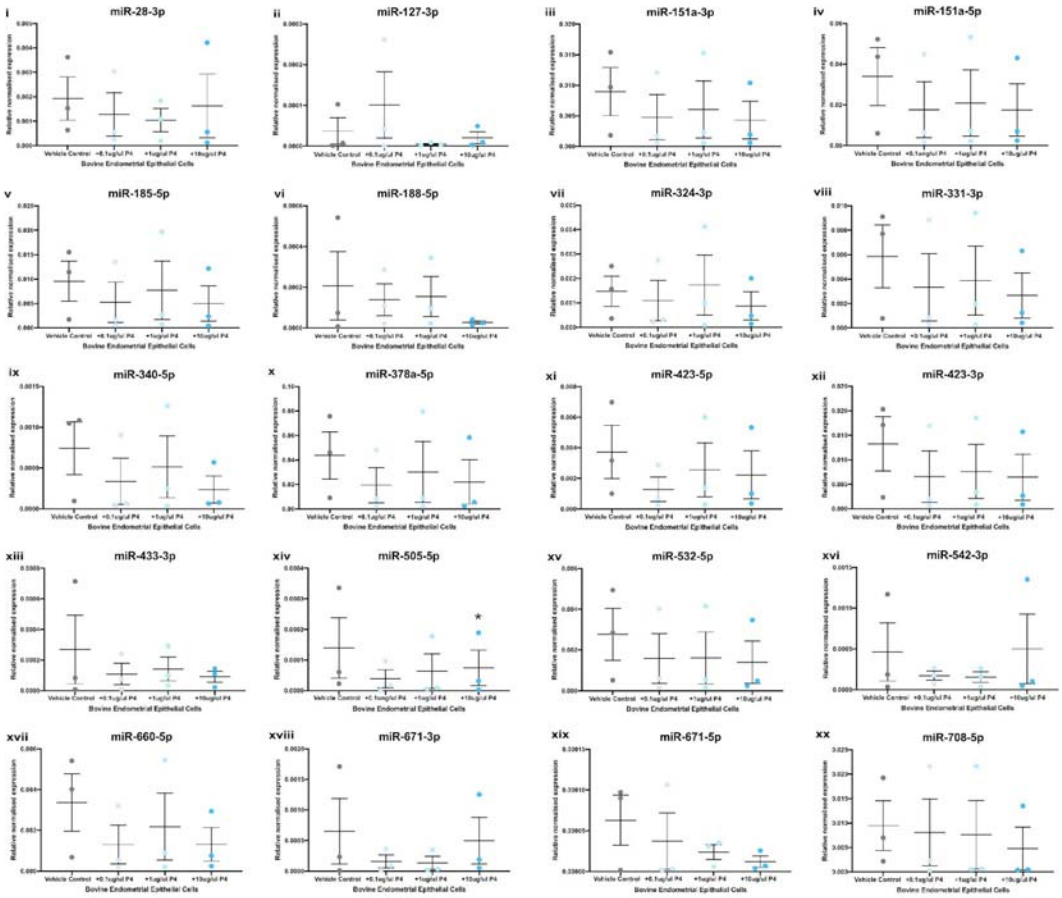

Figure 7. Expression of stem lineage miRNAs in bovine endometrial epithelial cells treated with P4. *Expression of stem lineage miRNA (i) miR-28-3p, (ii) miR-127-3p, (iii) miR-151a-3p, (iv) miR-151a-5p, (v) miR-185-5p, (vi) miR-188-5p, (vii) miR-324-5p, (viii) miR-331-3p, (ix) miR-340-5p, (x) miR-378a-5p, (xi) miR-423-3p, (xii) miR-423-5p, (xiii) miR-433-3p, (xiv) miR-505-5p, (xv) miR-532-5p, (xvi) miR-542-3p, (xvii) miR-660-5p, (xviii) miR-671-3p, (xix) miR-671-5p and (xx) miR-708-5p in bovine endometrial epithelial cells treated with vehicle control (grey circle), 0.1µg/mL (light blue), 1.0µg/mL (medium blue) or 10µg/mL P4 (dark blue circle) for 24 hours. Significant differences in miRNA expression values determined when p≤0.05 are depicted by an asterisk (*).*

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Figure 8:

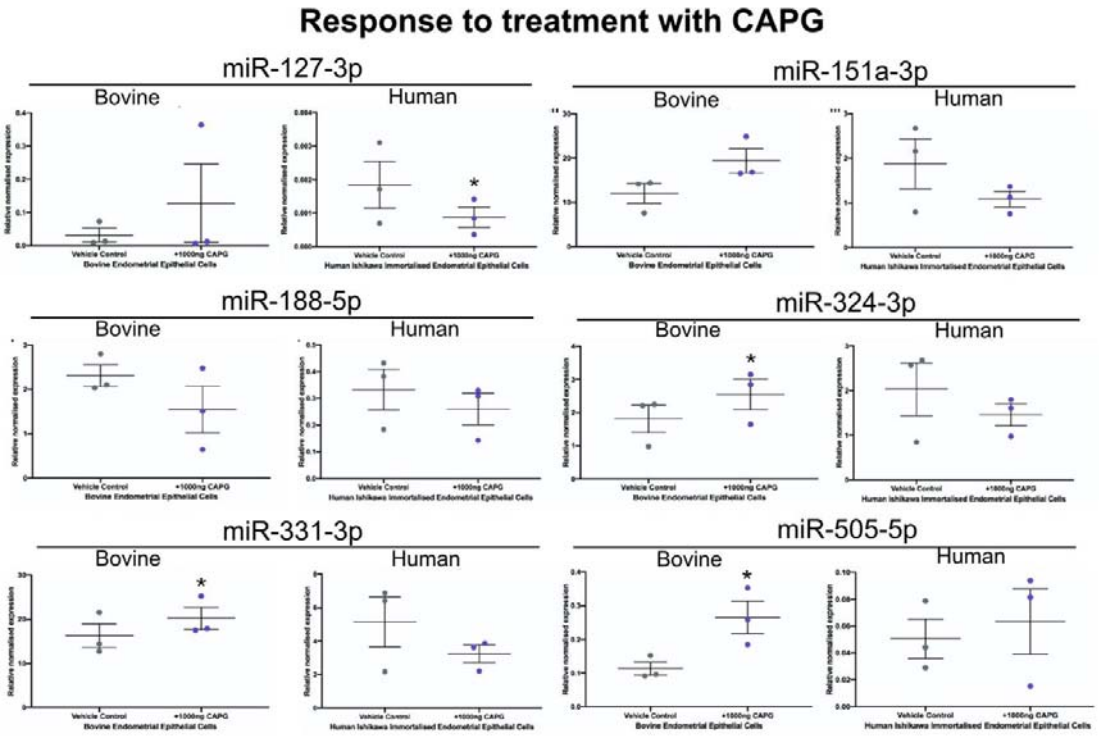

Figure 8. ***Regulation of stem lineage miRNAs in bovine and human endometrial epithelial cells treated with bCAPG.*** *Expression of stem lineage miRNAs miR-127-3p, miR-151a-3p, miR-188-5p, miR-324-3p, miR-331-3p, and miR-505-5p in either bovine (left hand side of each pair) or human (right hand side of each pair) endometrial epithelial cells. Primary bovine endometrial epithelial cells were treated with vehicle control (grey circle), or 1000ng/µl bCAPG (purple circle) for 24 hours. Human Ishikawa immortalized endometrial epithelial cells were treated with vehicle control (grey circle), or 1000ng/µl bCAPG (purple circle) for 24 hours. Significant differences in miRNA expression values determined when p≤0.05 are depicted by an asterisk (*).*

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Figure 9:

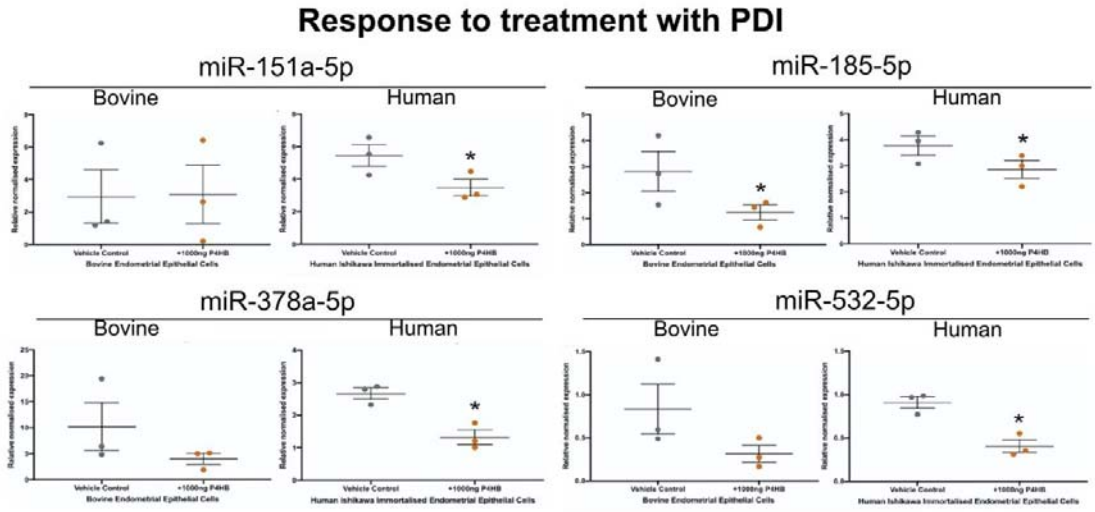

Figure 9: ***Regulation of stem lineage miRNAs in bovine and human endometrial epithelial cells treated with bPDI.*** *Expression of stem lineage miRNAs miR-127-3p, miR-151a-3p, miR-188-5p, miR-324-3p, miR-331-3p, and miR-505-5p in either bovine (left hand side of each pair) or human (right hand side of each pair) endometrial epithelial cells. Primary bovine endometrial epithelial cells were treated with vehicle control (grey circle), or 1000ng/µl bPDI (orange circle) for 24 hours. Human Ishikawa immortalized endometrial epithelial cells were treated with vehicle control (grey circle), or 1000ng/µl bPDI (orange circle) for 24 hours. Significant differences in miRNA expression values determined when p≤0.05 are depicted by an asterisk (*).*

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TABLES

Table 1: Vertebrate Species sampled, genome version, coverage and completion level.

Clade	Species	Version	Genome Quality
Fish	Zebrafish	GRCz11	Full Genome, Chromosome Level
	Chicken	Gallus_gallus-5.0	70X Coverage, Chromosome Level
Monotremes	Platypus	OANA5	6X Coverage, Chromosome Level
Metatheria	Opossum	monDom5	7.33X Coverage, Chromosome Level
Eutheria	Elephant	Loxafr3.0	7X Coverage, Scaffold Level
	Armadillo	Dasnov3.0	6X Coverage, Scaffold Level
	Mouse	GRCm38.p6	High Quality Reference Assembly
	Human	GRCh38.p12	High Quality Reference Assembly
	Mouse Lemur	Mmur_3.0	221.6X Coverage, Chromosome Level
	Bushbaby	OtoGar3	137X Coverage, Scaffold Level
	Cat	Felis_catus_9.0	72X Coverage, Chromosome Level
	Microbat	Myoluc2.0	7X Coverage, Scaffold Level
	Horse	Equ Cab 2	6.79X Coverage, Chromosome Level
	Cow	UMD3.1	9X Coverage, Chromosome Level

Table 1: *Set of 14 species sampled for the selective pressure analysis. Using Ensembl 92* (Yates *et al.*, 2016)*, a dataset of genomes representative of (i) vertebrate outgroup clades, or (ii) variations in placental morphology in metatherian and eutherian mammals. For each clade, genomes were chosen based on genome coverage for downstream homology searching. 'Clade' refers to the taxonomic group of each included species. 'Species' denotes the included species, by their common name. 'Version' denotes the genome assembly version included in this analysis. 'Genome Quality' refers to the coverage and assembly level of each species included in this analysis.*

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Table 2: Properties of each subject database used.

Dataset	Total Number of Sequences	Longest Sequence (base pairs)	Average Sequence Length (nt)
3'UTR	311524	122583	1021.9
5'UTR	337821	20453	232.1
Introns	6472686	4693080	4108.0

Table 2: *For each of the selected representative Vertebrate genomes, the 5'UTRs, 3'UTRs, introns and exons were accessed from Ensembl92* (Yates *et al.*, 2016). *These regions were constructed into subject datasets for homology searching in BLASTn2.6.0* (Altschul *et al.*, 1990)

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Table 3: Example of three functionally related proteins under positive selection on stem eutherian lineage

Family	Gene Name	lnL	*p*2	ω2	Positions in the alignment predicted to be positively selected.
PTHR13211	WRAP53	-10149.383	0.04821	37.80743	72, 88, 114, 225, 253, 260, 261, 331, 423, 480, 493, 506, 510, 513, 522, 528
PTHR12066	TERT	-36003.667	0.02302	954.86770	121, 122, 140, 166, 197, 508, 878, 1020, 1110
PTHR14487	ACD	-15296.106	0.10315	30.62227	138, 143, 148, 178, 185, 200, 202, 206, 208, 209, 214, 269, 278, 296, 298, 314, 347, 363, 368, 379, 390, 394, 395, 412, 414, 450, 470, 474, 524, 530, 532, 533, 545, 548, 561, 571, 575, 586, 685, 698

Table 3: *The panther family ID and common gene names are provided for a set of 3 proteins extracted to illustrate the cases of positive selection identified. The LnL value associated with the fit of the codeml model (PAML) to the data are provided as are the associated proportion of sites (p2) that have the corresponding ω2 (or Dn/Ds ratio). The sites estimated to be positively selected are given in the final column, these are numbered as per aligned codon position.*

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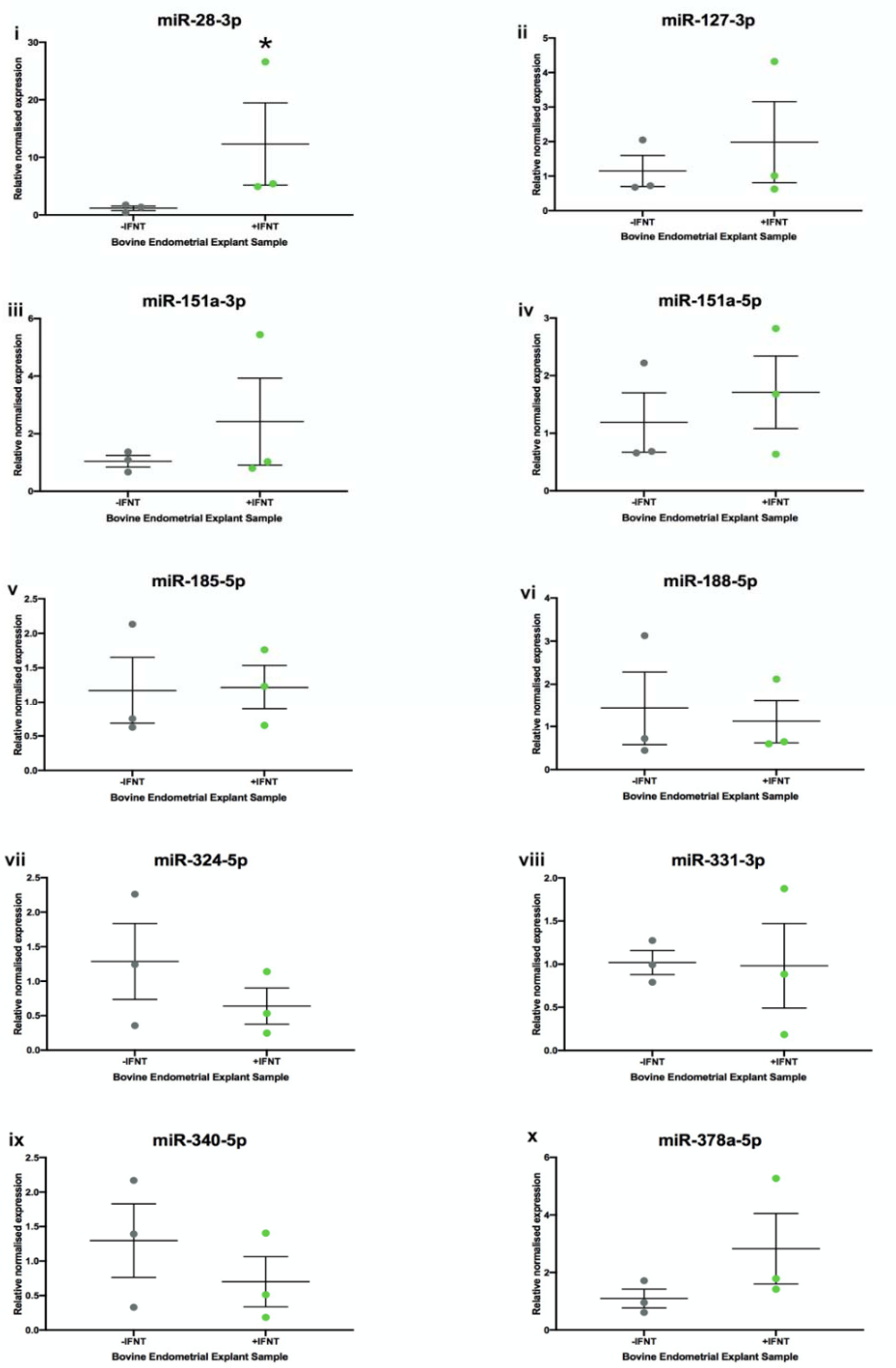
Supplementary Figure

Supplementary Figure 1:



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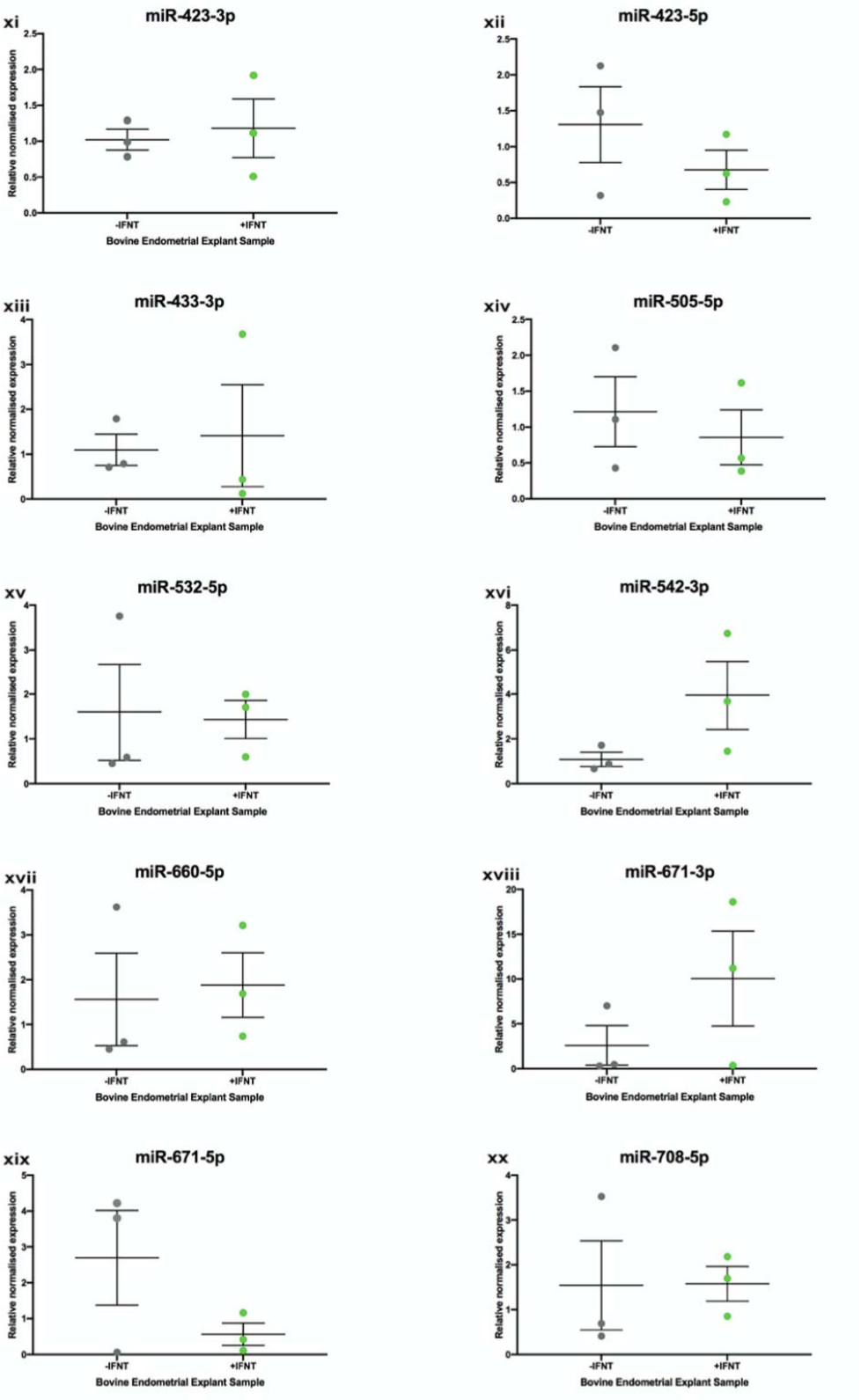

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Supplementary Figure 1. Expression of stem lineage miRNAs in bovine endometrial explants treated with recombinant oIFNT. Expression of stem lineage miRNA (i) miR-28-3p, (ii) miR-127-3p, (iii) miR-151a-3p, (iv) miR-151a-5p, (v) miR-185-5p, (vi) miR-188-5p, (vii) miR-324-5p, (viii) miR-331-3p, (ix) miR- 340-5p, (x) miR-378a-5p, (xi) miR-423-3p, (xii) miR-423-5p, (xiii) miR-433-3p, (xiv) miR-505-5p, (xv) miR-532-5p, (xvi) miR-542-3p, (xvii) miR-660-5p, (xviii) miR-671-3p, (xix) miR-671-5p and (xx) miR-708-5p in bovine endometrial explants treated with vehicle control (grey circle), or 1000ng/µl oIFNT (green circle) for 24 hours. Significant differences in miRNA expression values determined when $p<0.05$ are depicted by an asterisk (*).