DOI: 10.21451/1984-3143-AR997

Proceedings of the 31st Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Cabo de Santo Agostinho, PE, Brazil, August 17th to 19th, 2017.

Understanding the uterine environment in early pregnancy in cattle: How have the *omics* enhanced our knowledge?

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Abstract

Early pregnancy loss in cattle can be attributed to a myriad of sources. One key factor that can influence early pregnancy success or loss is the influence and interactions between the maternal environment and the developing embryo/conceptus. Recent advances in high-throughput 'omics' technologies coupled with improved bioinformatics capabilities represent a promising avenue for enhancing our understanding of fundamental developmental events – which would have direct agricultural, veterinary, and economic benefits. Thusly this review revolves around recent applications of advanced transcriptomic, proteomic, and metabolomic analyses within a bovine uterine secretomic and interactomic context, with an overriding aim to highlight the advantages of these emerging fields whilst identifying areas for improvement, consideration, and further research and development.

Keywords: phenomics, interactomics, proteomics, hormonomics, metabolomics, transcriptomics, epigenomics, genomics, uterine biology, reproductive physiology, maternal-embryo communication.

Introduction

Successful pregnancy of all species is contingent on a carefully orchestrated series of events along the reproduction continuum such that the foetus is born alive. A significant proportion of reproductive wastage occurs in the first 2-3 weeks of pregnancy (Bazer *et al.,* 2011). The initial obstacle is the successful deposition of the male gametes into the female reproductive tract, at the optimal time, with the objective to fertilise a high-quality oocyte, released from the ovulatory ovarian follicle. This ovulatory follicle subsequently undergoes a luteinising hormone (LH)-driven remodelling process to form a corpus luteum (CL), which produces progesterone (P4). This result is a post-ovulatory rise in P4 that is ordinarily sufficient for establishing an appropriate uterine environment, conducive to embryo growth and receptive to subsequent conceptus (embryo proper and extra-embryonic membranes) implantation.

Successful early embryo and conceptus development, however, is a bilateral process, requiring reciprocal signalling from the conceptus to the mother. Initial maternal recognition of pregnancy occurs on day 16 post-oestrus in cattle. Such reciprocality between mother and offspring is a complex and still poorly understood process but known to be influenced by maternal metabolic parameters in addition the quality of embryo present (Reviewed by Lonergan *et al*., 2016; Spencer *et al*., 2016).

One way of enhancing our understanding of early pregnancy loss is to elucidate the physiological biological and biochemical processes underpinning and regulating such fundamental reproductive events. Large scale 'omics' technologies offer scope for addressing many specific questions remaining around early pregnancy, and are addressed in this review.

The 'omics' suffix loosely describes the study of big data sets within functional biological niches, or the holistic study of intra-domain interactions, and is therefore considered high dimensional biology (Horgen and Kenny, 2011). This has been facilitated by advances in *en masse* sequencing technologies (Mochida and Shinozaki, 2011) and accompanying enhanced bioinformatic analysis capabilities (Gandomi and Haider, 2015).

Whilst the 'omics' have traditionally revolved around the central dogma of biology – genomics, transcriptomics, and proteomics, increasing attention is being afforded to multi-level inter-domain interactions, in addition to the emergence of additional domains, including epigenomics, interactomics, hormonomics, metabolomics, and phenomics. Figure 1 schematically depicts the hierarchical and overlapping nature of our current understanding of these 'omics' domains. All of the aforementioned are pivotal to understanding several fundamental reproductive processes and Fig. 2 schematically depicts various inter-domain interactions within an 'omics' context which underpin the formation and regulation of the uterine secretome.

This review focuses on the bovine and aims to summarise how these different 'omics' technologies have, and can, enhance our understanding of how the uterine environment successful supports early pregnancy, how the conceptus itself affects this bilateral communication, in addition to how factors in the maternal environment modify such interactions.

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Received: June 4, 2017
Accepted: July 14, 2017

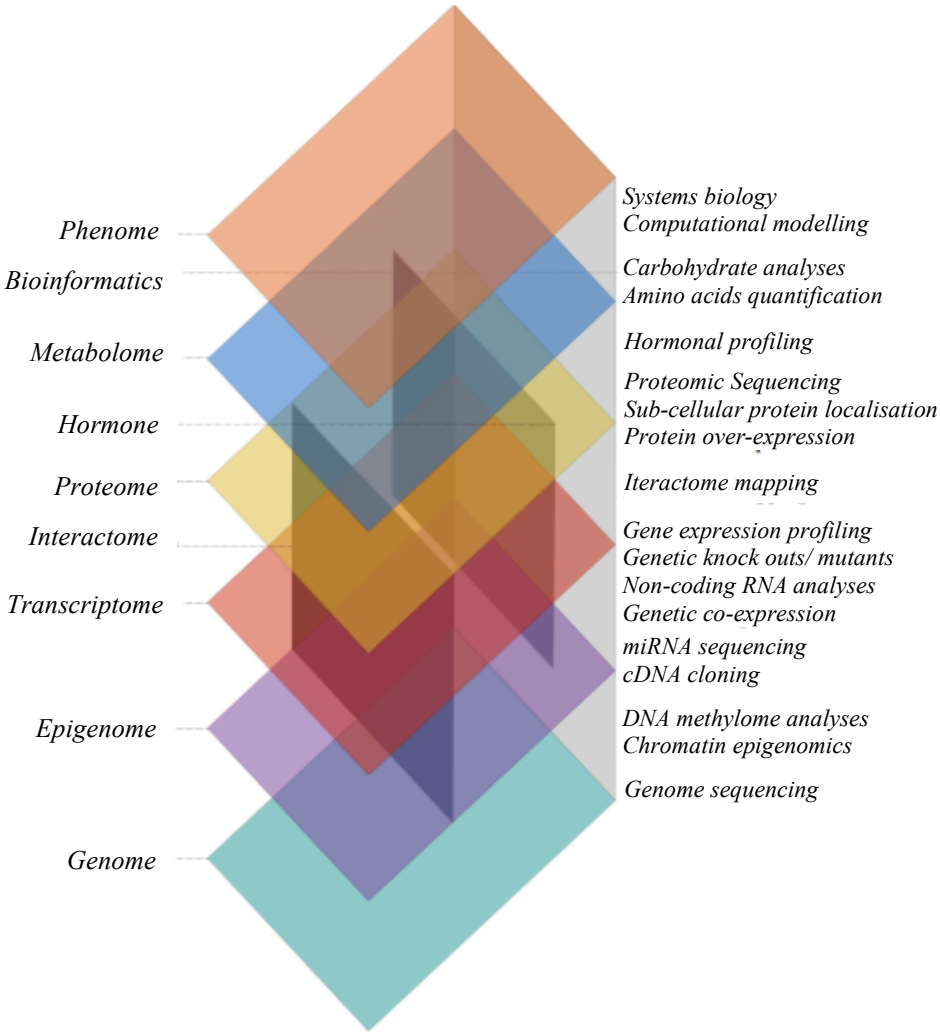

Figure 1. Multi-level linear relationship between 'omics' domains, with common corresponding available techniques for studying respective domains. Adapted from Mochida and Shinozaki (2011).

Uterine epithelial cell transcriptomics

Detecting changes due to steroid hormones

Initial reports of using transcriptomics to understand the physiology of the endometrium sought to determine the role that steroid hormones played in modifying gene expression patterns. Specifically, work by Bauersachs *et al.* (2005) compared endometria from day 0 of the oestrus cycle (low P4 and high oestradiol - E2) to that recovered from cycle animals on day 12 (dioestrus; high P4) to identify 133 differentially expressed genes. Additional studies determined that elevating (Forde *et al*., 2009) or decreasing (Forde *et al*., 2011a, 2012b) P4 in circulation advanced or delayed, respectively, the normal temporal changes that occur in the endometrium during the oestrus cycle. This altered the endometrial transcriptome and modified the elongation trajectory of the conceptus (Carter *et al*., 2008; Forde *et al*., 2011a) – a phenomenon previously demonstrated in cattle (Garrett *et al*., 1988). More recently, RNA sequencing has been used to identify how ovulation of different sized follicles (and the different endocrine environments that the endometrium is therefore exposed to), alters the endometrial transcriptome (Mesquita *et al*., 2015).

Transcriptomic changes induced by the conceptus

Dating back to the late 1970s and early 1980s it was understood that conceptus presence has an anti-leutolytic effect on the CL and this was required to occur by day 16 following oestrus *i.e*. day 16 was the day of pregnancy recognition (Betteridge *et al*., 1980; Northey and French, 1980). It was later identified that a secretory protein component of the conceptus could inhibit uterine prostaglandin F2 alpha (PGF2α) production as well as extend CL formation (Knickerbocker *et al*., 1986a, b) and was confirmed as a type 1 interferon, interferon tau (IFNT; Helmer *et al*., 1989). Prior to the advent of large-scale transcriptomics, most effects of the conceptus on the endometrium, targeted alterations to candidate genes involved in the prostaglandin pathway (both production and inhibition) as well as candidate classical interferon stimulated genes (ISGs) (Reviewed in Forde and Lonergan, 2012). The initial work in the bovine endometrium was performed using microarray technology using a combination of both 'in-house' (Bauersachs *et al*. , 2006; Klein *et al*., 2006; Mansouri-Attia *et al*., 2009a)

and commercially available arrays used (Forde *et al*., 2011b; Walker *et al*., 2010). As with the candidate gene approach, the majority of changes induced by the conceptus were classical ISGs.

Interestingly, Gene Set Enrichment Analysis (GSEA) performed on the lists of differentially expressed transcripts from these different studies using different platforms (in-house *vs*. affymetrix™), performed in different laboratories, on different samples (days, 12, 15, 16, 18, and 20 of confirmed pregnant compared to cyclic heifers) identified a consistent transcriptomic signature in the endometrium associated with the presence of the conceptus during the peri-implantation period of pregnancy in cattle (Bauersachs *et al*., 2012). More recent uses of RNA sequencing (a more sensitive technique) have identified that milder embryo/conceptus induced changes are detectable prior to pregnancy recognition on day 13 (Forde *et al*., 2012a) with some as early as day 6 (Binelli *et al*., 2015).

Intriguingly two separate studies also identified a transcriptomic signature as early as day 18 of pregnancy in the intercaruncular endometrium that is specific to the type of conceptus present. Even on day 18 of pregnancy, the endometrium responds differently to embryos with known different developmental outcomes and modifies its response to IVF-produced conceptus *vs*. cloned conceptus (Bauersachs *et al*., 2009). This altered endometrial transcriptomic response is magnified by day 20, particularly in the caruncular region of the endometrium (Mansourri-Attia *et al*., 2009a). More recently, attempts were made to access whether the endometrium alters its response to male compared to female conceptuses (Forde *et al*., 2016). However, despite there being a large component of sexual dimorphism both at the blastocyst stage (Bermejo-Alvarez *et al*., 2010) and during the peri-implantation period (Forde *et al*., 2016) no appreciable difference in the endometrial transcriptomic response was detected between male and female conceptuses. This may indicate that the endometrium does not 'favour' one over the other is simply due to a non-transcritomic response yet to be identified.

The uterine secretome

The aforementioned work describing endometrial transcriptomics changes have enhanced our understanding of the temporal changes that occur in the endometrium as well as how the conceptus alters expression patterns. Such transcriptomic changes however are not always reflected further up the 'omics' pipeline, *i.e*. those mRNA changes that we observe in the endometrium are not always translated into changes in the protein content of the uterine luminal fluid (ULF) or the metabolic molecules that comprise the ULF. These collectively make up the uterine secretome – a spatiotemporally dynamic secreted, or actively transported, milieu in which the free-floating conceptus is bathed and which is required for growth of the conceptus past the hatched blastocyst stage of development (Gray *et al.,* 2001; Brandão *et al.,* 2004; Alexopoulos *et al.,* 2005).

As depicted by Fig. 2, the uterine secretome, also known as ULF or histotroph, composition is influenced by four parameters. Whilst the vasculature (*i*) contributes a plethora of factors including ovarian sex hormones (Einer-Jensen and Hunter, 2005) and immune cells (Singh *et al*., 2008; Healy *et al*., 2014; Sheldon *et al*., 2014), uterine secretions are predominantly produced by the (*ii*) uterine glandular and (*iii*) epithelial cells. The physiological conceptus (*iv*), if present, also secretes factors which alter the microenvironmental consistency. The nature of such uterine secretions is largely transcriptomic (miRNAs), metabolic (carbohydrates and amino acids), and proteomic (enzymes and signalling molecules), and are discussed below.

Secretome proteomics

Previous studies looking at the composition of bovine ULF have been limited, not only by access to the sample matrix aforementioned, but also by antibody availability. Recent advances, particularly in mass spectrometry (and in label free quantitative analysis of protein composition such at iTRAQ analysis) have advanced 'omics' technologies to enable the evolution from analysing whole ULF composition for basic constituents, including proteins and metabolites (discussed below), towards wholesale temporal screening of complex uterine and embryo derived exosomes, or nanovesicles (Saadeldin *et al*., 2015; Campoy *et al*., 2016).

Similarly to the temporal changes observed in the endometrial transcriptome, iTRAQ analysis of the ULF from different stages of pregnancy identified a temporal change not just in the composition of proteins in the ULF, but also in the quantity of specific proteins (Forde *et al*., 2014a). However, there is an order of magnitude in the difference in the number of transcripts detected compared to the numbers of proteins – which may reflect technological limitations as opposed to a true biological phenomenon. In addition, there are considerably more proteins present, or being produced, by the conceptus on day 16 of pregnancy (Forde *et al*., 2015) than IFNT alone. Transcriptomic analysis of genes differentially expressed due to the presence of the conceptus are similar but not solely attributable to IFNT production (Bauersachs *et al*., 2012) with previous studies in both cattle (Bartol *et al*., 1985) and sheep (Brooks *et al*., 2014) determining that additional molecules modify the endometrium during the time of pregnancy recognition including cortisol and prostaglandins. Therefore, it is likely the bovine conceptus also produces proteins in addition to IFNT that modify endometrial function, and may not always be secretory in nature (Forde *et al*., 2015).

Recently, extracellular vesicles (EVs) have emerged as a non-traditional form of cell-to-cell communication (paracrine signalling). EVs are membrane-bound vesicles (classified by size) and contain microRNAs (miRNA), proteins, as well as other RNA molecules capable of being incorporated into target tissues (Raposo and Storvel, 2013). One of the first reports of this phenomenon in the uterus was

through the incorporation of endogenous retroviral envelope proteins that were shed from the endometrial epithelium and incorporated into the trophectoderm cells of sheep conceptuses, with more recent data confirming that the mode of transfer of these retroviral particles was via exosomes (Black *et al.,* 2010; Burns *et al*., 2014, 2016). This phenomenon has been reported in other species *e.g*. sheep and cattle at different points on the reproductive axis (Machtinger *et al*., 2012). It is likely that EVs pay a role in bovine conceptus-maternal communication via RNA species, including miRNA, transport. Detailed analyses of the composition of EVs is now possible with advances in mRNA, miRNA and protein sequencing.

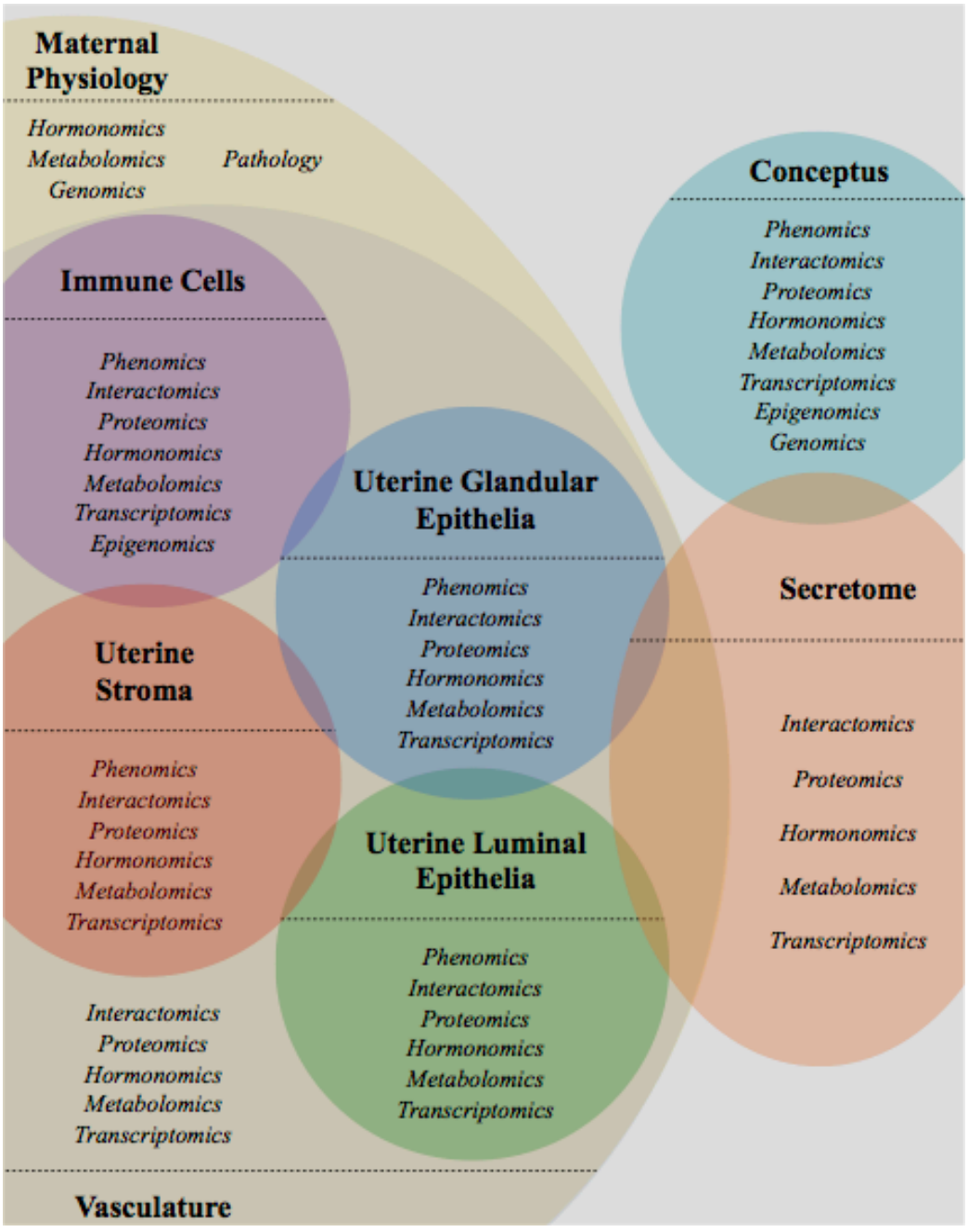

Figure 2. Schematic representation of the constituents which contribute to the bovine uterine secretome. Overlapping depicts the influence each constituent has on one another — the secretome is primarily influenced by uterine glandular and luminal epithelia in addition to conceptus presence, and to a lesser extent, leak-through from the maternal vasculature, whose composition is in turn influenced by maternal physiology. For a comprehensive review on embryo 'omics' please refer to Krisher *et al*. (2015).

Secretome metabolomics

Aside from passive fluid seepage into the uterine lumen from the oviducts, cervix, vagina, and the ejaculate post-intercourse, histotroph composition is largely attributable to, and regulated by, the uterine luminal and glandular epithelia, thus considered the uterine lumen gatekeepers (Leese *et al*., 2008). Combined with the vast transcriptomic data from these gatekeepers described, providing a clearer picture of the

spatiotemporally dynamic behaviour of these cells, the evolution in metabolic profiling analyses (cheaper, quicker, more robust, and more sensitive) has enabled a shift away from analysing whole uterine luminal fluid for basic constituents – such as pH (Hugentobler *et al*., 2004), ions (Hugentobler *et al*., 2007b), amino acids (Hugentobler *et al*., 2007a), carbohydrates (Hugentobler *et al*., 2008), and single enzymes, such as N-acetyl-beta-D-glucosaminidase (Hussain *et al*., 1989) – on a few days and in a few cows, towards comprehensively profiling the uterine fluid of a plethora of animals and on numerous days, whilst simultaneously comparing and contrasting this data between animals with varying physiologies and metabolic statuses.

Within the context of maternal-conceptus interactions, for example, whilst the importance of amino acids for embryo development *in vivo* and *in vitro* have been well established (Morris *et al.,* 2002; Wirtu *et al*., 2003; Sturmey *et al.,* 2010; Wale and Gardner 2010; Leese 2012), and has been comprehensively studied in the sheep (Gao *et al*., 2009), little was known about the basic requirements for conceptus elongation and successful pregnancy recognition in cattle. For this reason Forde *et al*. (2014b), in brief, analysed *en masse* the amino acid composition of beef heifer uteri which had been inseminated (n = 59) *vs*. a non-inseminated cyclic control group (n = 24) on days 7, 10, 13, 16 or 19 of pregnancy and days 7, 10, 13, or 16 of cyclic animals. Whilst several differences between the two groups were observed, when both pregnancy and day effects were considered, only differences in threonine, glutamate, and valine flux in ULF were observed, thus offering scope for targeted experimentation into the role of these amino acids in day 7-19 embryo development. Moreover, the total amino acid content of ULF increased as the blastocyst progressed to an elongated filamentous conceptus, most likely to accommodate for the greater metabolic demands of the developing offspring (Souza *et al.,* 2015). Interestingly, this data loosely corroborates earlier findings that total ULF amino acid concentrations are lower in sub-fertile animals (Meier *et al.,* 2014) and in cattle carrying developmentally compromised (cloned) embryos (Groebner *et al.,* 2011).

As aforementioned, transcriptomic advances – such as endometrial transcriptomic profiling at day 20 being predictive of the type of conceptus present (*in vivo vs. in vitro* derived *vs.* cloned; Bauersachs *et al.,* 2009; Mansouri-Attia *et al.,* 2009b) – led to the question of whether the same was the case for male *vs.* female conceptuses, and if such transcriptomic changes were reflected in the amino acid composition of the respective ULF. Thus Forde *et al*. (2016) inseminated 30 heifers prior to uterine and conceptus recovery on day 19 following oestrus. Their data showed that 9 amino acids (asparagine, histidine, glutamine, arginine, tryptophan, methionine, phenylalanine, isoleucine, and lysine) were present in higher levels in the ULF of XY containing uteri *vs.* XX positive counterparts however, there was no altered endometrial transcription of amino acid transporters observed. This interactomics inconsistency suggests that the amino acid consumption of XX *vs.* XY conceptuses is different whilst ULF secretions are constant. In support of this, Sturmey *et al.* (2010) demonstrated that male bovine blastocysts consumed less amino acids and exhibited a lower amino acid turnover compared to female blastocysts.

A significant challenge to the dairy industry is cattle sub-fertility arising from a systemic negative energy balance (NEB). Data show that a NEB perturbs endometrial transcriptomics (Cerri *et al.,* 2012), however unclear was whether lactation status influences ULF composition. To this end Bauersachs *et al*., 2017 (Forde 2017; Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, Leeds, UK; unpublished data) analysed the amino acid and carbohydrate composition of ULF from lactating (n = 6) *vs.* non-lactating (dry) cows (n = 7) *vs.* maiden heifers (n = 4). Glutamate was the only differentially transported amino acid, lower in lactating cows relative to heifers but not dry cows. Conversely, lactate was higher in the lactating cohort compared against heifers but not relative to dry cows. Glucose and pyruvate remained unchanged. A potential aetiology for this inconsistency between transcriptomic and secretomic data, however, may lie in the fact that the heifer group was artificially inseminated (AI) whereas lactating and dry animals had embryos transferred. In other words, conceptus origin (AI *vs.* transfer) may have a greater impact on ULF composition than maternal lactating physiology.

An interesting supplementary observation arising from the *en masse* data analyses aforementioned was that the ULF from ipsilateral uterine horns comprised more glycine than those contralateral on day 19 of pregnancy Forde *et al*., 2017 (Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, Leeds, UK; unpublished data). Groebner *et al.* (2011) too reported an amino acid abundance increase in the ipsilateral horn on day 18, suggestive that conceptus presence stimulates differential amino acid flux into the lumen. A second hypothesis worthy of further investigation is that the ipsilateral endometrium is exposed to more P4 released from the large luteal cells of the corpus luteum therein signalling greater glycine secretion into the ipsilateral uterine horn.

Additional considerations

It is worth noting that, unlike in the human (Cheong *et al*., 2013), obtaining bovine ULF aspirates is unfeasible owing to minuscule volume, and viscous consistency, of physiological ULF. Thus the field has been reliant on studying the composition of ULF by flushing tracts. Advantages of flushes include the recovery of ample volumes for subsequent 'omics' analyses (Velazquez *et al*., 2010). On the other hand, the greatest disadvantage is perhaps the inability to calculate dilution coefficients precisely as less fluid is recovered than injected into the uterine horn. Additional robust sampling methodologies are available such as the direct ULF sampling via uterine exteriorisation and catheterisation of anaesthetised heifers (Hugentobler *et*

al., 2007a), which also have associated advantages and disadvantages. For a comprehensive review pertinent to the relative merits of various complementary reproductive tract sampling technologies please refer to Leese *et al*. (2008) and Velazquez *et al*. (2010).

Another potentially fruitful area for future work resides in telocytes – a recently discovered cell type (Popescu and Faussone-Pellegrini, 2010) identified and characterised in the human myometrium (Ciontea *et al*., 2005), endometrium (Cretoiu *et al*., 2013), rat endometrium (Hatta *et al*., 2012), and recently the bovine oviduct (Abd-Elhafeez and Soliman, 2017), though not in the bovine uterine environment yet. Telocytes are reported to secrete exosomes (45 ± 8 nm), ectosomes (128 ± 28 nm), and multi vesicular cargos (MVCs; 1 ± 0.4 μm; Roatesi *et al*., 2015) and thus may – indirectly, through paracrine interactions with the stroma and epithelium, and directly, via contributing to the uterine secretome – play a role in maternal-embryo dialogue (Saadeldin *et al*., 2015; Campoy *et al*., 2016).

In the wider context of reproductive 'omics' an interesting recent development is the identification that miRNA molecules in circulation in bovine plasma can be used to identify pregnancy as early as day 8 (Ioannidis and Donadeu, 2017). Utilising such RNA sequencing capabilities for elucidating biomarkers for identifying potentially subfertile pathophysiologies in cattle, as in the human (Sathyapalan *et al*., 2015), will be an important development for both selectively mating, inseminating, or embryo transferring into heifers, in addition to enhancing our holistic understanding of fundamental reproductive events (Fig. 2).

There is a "clear need for a new means of interrogating the intrauterine environment" (Cheong *et al.,* 2013). Owing to the technical, and often ethical, limitations surrounding *in vivo* sampling, one direction towards addressing this includes *in vitro* modelling. Advances have been made regarding the production of bovine *in vitro* derived oviduct fluid (*iv*DOF) which resemble *in vivo* derived fluid (Simintiras and Sturmey 2017; Simintiras *et al.,* 2017) and moreover supports early embryo development (Chen *et al*., 2017), however, this can be considered currently under optimisation and has yet to be recapitulated to the uterus. The delay in an *in vitro* uterine luminal fluid (*iv*ULF) is partly attributable to the increased complexity of the endometrium, notably the presence of glands and a greater number of different cell types. Thus, for the time being ULF must be obtained using *in vivo* and *ex situ* techniques.

Conclusions

What is clear from the application of these large-scale transcriptomic analyses of concept-maternal interactions is that significant advances in the field can be made. Nonetheless there are obstacles to utilising these technologies. Firstly, International standards for best practice, in addition to the free sharing of such big data, is critical to the sound interpretation of resulting omics analysis. Secondly, the integration of the data generated from these various 'omics' platforms, although advancing, is still not fully integrative from an interactomics perspective. Last but not least, sound interpretation of such data relies on a cognisant understanding of the biological question being asked – *i.e*. whether one is using these technologies as a hypothesis generating tool, or to further understand the non-canonical roles and factors central to successful early pregnancy.

Acknowledgements

Supported by Science Foundation Ireland (13/IA/1983) and by the European Union Seventh Framework Programme FP7/2007-2013 under grant agreement n° 312097 ('FECUND').

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