Project title: Examining the myometrial transcriptome in twin pregnancy
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Background
The incidence of preterm birth (<37 weeks) among twin pregnancies is approximately 50% compared to ~10% in singleton pregnancies\(^1\). There is an urgent need for a better understanding of why twins are at high risk of preterm delivery as well as developing better modes of treatment and prevention\(^2\). Key to this is determining the differing pathophysiological mechanisms of preterm birth in this population\(^3\) including the role of the myometrium in spontaneous preterm delivery.

Premature activation of uterine contractions underlies spontaneous preterm labour. These contractions arise from either perturbations in the physiological process of contraction itself or from pathological factor(s) initiating them\(^4\). In multiple pregnancy, the aetiology of preterm birth in twins is likely to be multifactorial and different to that of a singleton pregnancy. Factors identified for preterm birth in twins include uterine overdistention. Stretch of the myometrium increases its contractility through upregulation of prostaglandin synthesis and release and oxytocin receptor expression\(^5\) which together contribute towards the cascade of events leading to labour onset.

In controlled in vitro conditions, we have shown significant differences in contractility between myometrium from singleton and twin pregnancies\(^6\) and shown reduced potency of some tocolytics in twins\(^7,8\). We proposed that differences in myometrial gene expression underlie these changes.

The aims of this study were to examine for differences between the transcriptome of myometrium from singleton and twin pregnancy (matched for gestational age at term and preterm), to identify differentially expressed genes between the two groups and the biologically important pathways which are enriched for them.

Methodology
Women undergoing elective pre-labour CS with either singleton or twin pregnancy were recruited in pre-op clinic to donate a myometrial biopsy to the Liverpool Women’s Research Tissue Bank. Samples were comprised of myometrium taken from the upper lip of the lower uterine incision site. Women were eligible for inclusion if they were delivering preterm (<37 weeks) or term (between 37 and 39 weeks). Samples were matched for gestational age (within each age group) BMI, and maternal age and categorised into 4 groups (n=6 women per group): Singleton preterm (SP), twin preterm (TP), singleton term (ST) and twin term (TT).

All samples were obtained with informed written consent prior to surgery. The study was approved by the local research ethics committee (REC ref 10/H1002/49).

RNA was extracted using standard laboratory methods (Trizol and RNA isolation kit). RNA integrity was assessed using an Agilent Bioanalyzer. RNA with an integrity number (RIN) of 7 or above was considered acceptable.
Transcriptomics analysis of myometrium from singleton and twin pregnancies at term or preterm was performed by RNA-sequencing at the University of Liverpool’s Centre for Genomic Research (CGR) facility. Total RNA underwent PolyA-selection, cDNA synthesis, fragmentation and indexing to create 24 RNASeq libraries which were prepared using a protocol for Illumina technology. Paired-end sequencing was performed on the Illumina HiSeq 4000 platform using sequencing by synthesis chemistry to generate 2 x 150bp paired-end reads. Mapping and alignment of reads to the human reference genome (Ensembl GRCh38.dna) was performed using TopHat2 version 2.1.0\textsuperscript{9}. Reads were counted according to the gene features which they mapped to using HTSeq-count version 0.6.1p1\textsuperscript{10}. Identification of differentially expressed genes was performed using DESeq2 package in R software\textsuperscript{11}. Gene Ontology (GO) functional term and Kyoto Encyclopaedia of Genes and Genomes (KEGG) response pathway analysis was performed using the R package ‘gage’\textsuperscript{12}.

Four contrasts were performed:

1. Singleton Preterm (SP) vs. Twin Preterm (TP)
2. Singleton Term (ST) vs. and Twin Term (TT).
3. Singleton Preterm (SP) vs. Twin Term (TT)
4. Singleton Term (ST) vs. Twin Preterm (TP)

Contrasts 1 and 2 were performed to identify differences between singleton and twin myometrium when matched and controlled for gestational age. Contrast 3 was performed to loosely look for the effects of uterine over distension and contrast 4 was performed to act as control for contrast 3.

**Key milestones**

- Recruitment of patients and collection of myometrial biopsies – completed 30\textsuperscript{th} Nov 2017
- RNA extraction and quantification of 24 samples completed - Dec 11\textsuperscript{th} 2017
- Samples submitted to Centre for Genomic Research, 14\textsuperscript{th} Dec 2017
- Initial sample QC completed – 12\textsuperscript{th} Jan 2018
- Recruitment of 2 further patients, RNA extraction and submission of replacement RNA – 19\textsuperscript{th} July 2018
- QC outcome of final 24 sample cohort selected– 20\textsuperscript{th} Aug 2018
- Preparation of 24 RNASeq libraries for Illumina protocol and paired end sequencing of 24 indexed libraries on the Illumina HiSeq 4000 platform – completed 19\textsuperscript{th} Sept 2018
- Data processing begins – 20\textsuperscript{th} Oct 2018
- Read alignment and differential gene expression analysis complete – 23\textsuperscript{rd} Nov 2018
- GO term and KEGG pathway analysis complete – 30\textsuperscript{th} Nov 2018
- Data interpretation - ongoing

**Progress so far**

Data output was received at the beginning of December 2018, we are currently in the process of examining the outcomes to be able to make meaningful interpretations of it.

In an initial interrogation of data sets, the numbers of differentially expressed genes identified between singleton and twin myometrium when matched for gestation (SP vs. TP and ST vs. TT) are
small, suggesting little or no difference between the myometrial transcriptome of the two groups. GO term and KEGG pathway analysis may yield more meaningful data.

Contrast 3 comparing SP vs. TT to loosely examine for the effects of uterine overdistention identified 99 genes which were differentially expressed. Interestingly, there was only one gene which was differentially expressed in contrast 4, suggesting that the transcriptome of singleton term myometrium is similar to that of preterm twin myometrium. Again we are currently examining the functional pathway analysis to determine which biological functions (if any) are up or down regulated in twin pregnancy.

We aim to have a final report available mid 2019 and plan to present and publish findings at international and national conferences and peer reviewed journals.

Challenges

This study involved the collection of myometrial biopsies form women undergoing planned CS with singleton or twin pregnancy. We have a dedicated twin clinic at Liverpool Women’s hospital hence women with twin pregnancy are seen more regularly than those with uncomplicated singleton pregnancy and were identified and recruited more quickly. Delay in recruitment was from identifying women with singleton pregnancy delivering preterm by elective CS (i.e. medically indicated preterm birth for maternal-fetal indications) to match the gestation of those with twins. Nevertheless, initial recruitment was completed by December 2017.

RNA was extracted and sent to Liverpool’s Centre for Genomic Research facility for QC and bioanalysis on schedule in December 2017. Of the 24 samples forwarded to the CGR, two were found unsuitable for sequencing (RIN <7), prompting the need for further recruitment, sample collection and extraction. This occurred at the time the main researcher, Dr Sarah Arrowsmith went on maternity leave. This resulted in a short delay in getting the final sample cohort to the facility for sequencing. The final sample cohort was collected, processed and ready for sequencing by mid July 2018, approximately 6 months later than planned.

Planned output


Presentation of the results at a national conferences (e.g. BMFMS)

Publication of the study findings; Comparison of the myometrial transcriptome from singleton and twin pregnancies by high throughput RNA-Seq.

References
