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1 Altered endometrial immune gene expression in beef heifers with retarded embryos

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14 Abridged title: Altered endometrial immune gene expression in beef heifers.

16 **Abstract**

17 The objective was to compare endometrial gene expression profiles in a group of beef
18 heifers yielding viable or degenerate embryos on Day 7 after oestrus as a means to
19 potentially explain differences in embryo survival rates. The focus was on genes that were
20 associated with either the pro- or anti-inflammatory immune response. Endometrial gene
21 expression was determined using q-RT-PCR analysis. Expression of Beta defensin
22 (*DEFB*), Interferon alpha (*IFN α*), Interferon gamma (*IFN γ*), Interleukin 6 (*IL6*),
23 Interleukin 10 (*IL10*), Forkhead box P3 (*FOXP3*) and Natural cytotoxicity triggering
24 receptor 1 (*NCRI*) was lower in endometria from heifers that produced viable embryos
25 compared with those that produced retarded embryos. Expression of Nuclear factor of
26 kappa light polypeptide gene enhancer in B-cells 1 (*NKFB1*), Transforming growth factor
27 beta (*TGF β*), Interferon gamma-inducible protein 16 (*IFI16*) and Interleukin 21 (*IL21*)
28 was higher in viable than in retarded heifers. We propose that small disturbances in the
29 expression of immune genes in the endometrium on Day 7 after estrus can have
30 detrimental effects on embryonic survival.

Introduction

In a previous study (Beltman *et al.*, 2010) the relationship between systemic (endocrine/physiological) and uterine (endometrial gene expression) dysfunction in the initial period of embryonic development from fertilisation to blastocyst formation in subfertile beef heifers was investigated. Two genes, Lysozyme 2 (*LYZ2*) and Ubiquitin-like with PHD and ring finger domains 1 (*UHRF1*) that are closely associated with the regulation of the immune system were increased in expression in the endometrium of heifers that yielded retarded embryos on Day 7. Tight regulation of the immune system is required as increased inflammatory cytokines disrupt the hypothalamic-pituitary-gonad axis (Hansen *et al.*, 2004). At a local level, regulation of the immune system in the endometrium is already evident as early as day 7 of pregnancy (Low *et al.*, 1990) and is critically important in pregnancy recognition as well as facilitating implantation (Forde *et al.*, 2010; Mansouri-Attia *et al.*, 2009; Walker *et al.*, 2010; Bauersachs *et al.*, 2012). Because embryo development is dependent on a tight regulation of the maternal immune system (Hansen, 1997; Leung *et al.*, 2000; Hansen, 2011), expression of components of the immune system in the endometrium could be associated of successful or unsuccessful embryonic development. Therefore, the aim of this study was to document the the expression of a broad range of immune-related genes in the endometrium of heifers producing viable and degenerate embryos.

Materials and Methods

All experimental procedures involving animals were approved by the University's Animal Research Ethics Committee and were licensed by the Department of Health and Children,

Ireland, in accordance with the Cruelty to Animals Act (Ireland 1876) and European Community Directive 86/609/EC.

Animal management and treatments

The experimental design used for this study was as previously described (Beltman *et al.*, 2010). Oestrus was synchronised in cross-bred beef heifers (n=157) using a Controlled Intravaginal Drug Releasing device (CIDR) protocol. Heifers detected in standing estrus (within 24-48 h post CIDR removal, n=102) were inseminated (o estrus=Day 0) with frozen-thawed semen from a single ejaculate of a bull of proven fertility. Tissue collection took place at slaughter on Day 7 post-oestrus. Heifers from which an embryonic structure was recovered were classified as either (i) viable, when the embryo was at the correct developmental stage (i.e. morula/early blastocyst), or (ii) retarded, when the embryo was arrested at the 2- to 16-cell stage. Heifers from which an unfertilised oocyte was recovered or from which no structure was recovered were omitted from the study. Strips of endometrial tissue from the uterine horn were processed stored at -80°C prior to RNA extraction.

Quantitative real-time PCR (q-RT-PCR)

Quantitative real-time PCR (q-RT-PCR) was performed on candidate genes identified in the literature as being involved in the immune response (Chapwanya *et al.*, 2009; Eckersall and Bell, 2010). In order to characterise possible mechanisms leading to the up- or down-regulation of the initially identified genes, a further 8 genes were chosen in order to further identify these.

RNA extraction and quantification was performed as described in the previous paper by Beltman *et al* (2010).

All primers were designed using Primer BLAST online software and manufactured by Eurofins MWG (Ebersberg, Germany). qPCR was carried out on the 7,500 Fast Real-Time PCR System (Applied Biosystems, USA). Each reaction consisted of 20 ng cDNA, forward and reverse primers at the optimised concentrations, 10 µl SYBRgreen mastermix (Applied Biosystems, USA) with a final reaction volume of 15 µl made up with RNase- and DNase-free water.

All reactions were carried out in duplicate and cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min and were carried out with the inclusion of a dissociation curve to ensure specificity of amplification. A standard curve was included for each gene to generate arbitrary expression values for all genes examined. Qbase plus software was used to perform a geNorm study to determine the most appropriate reference gene for our model system (Vandesompele *et al.*, 2002). The optimal number of reference targets in this experimental situation was determined as 3 (geNorm $V < 0.15$ when comparing a normalisation factor based on the 3 or 4 most stable targets). As such, the optimal normalisation factor was calculated as the geometric mean of reference targets *ACTB*, *RPL19*, and *PPIA*. All expression data for genes of interest are expressed as mean calibrated normalised relative expression values in arbitrary units (CNRQ values). Significant differences in gene expression between groups were determined by a Students t-test (Snedecor and Cochran, 1989) when the *P* value was < 0.05 .

Results

Following flushing of uteri, structures (oocytes/embryos) were recovered from 64% of the heifers of which 32 were classified as viable (i.e. morula/early blastocyst stage of development) and 19 were classified as retarded (i.e. arrested at 2- to 16-cell stage of development). The remaining recovered structures (n=14, 14%) were single-celled unfertilised oocytes and uterine tissues from these heifers were then omitted from the study.

The results of the qRT-PCR analysis are displayed in Figure 1 and 2. Eleven genes were significantly differentially expressed in the endometrium of heifers yielding viable compared with retarded embryos. Of these 11 genes, the expression of Beta defensin 1 (*DEFB1*), Interferon alpha (*IFNA*), Interferon gamma (*IFNG*), Interleukin 6 (*IL6*), Interleukin 10 (*IL10*), Forkhead box P3 (*FOXP3*) and Natural cytotoxicity triggering receptor 1 (*NCR1*) was significantly lower ($P<0.05$) in the endometrium of heifers from which a viable embryo was recovered compared with those yielding a retarded embryo. In contrast, endometrial expression of Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NKFB1*), Transforming growth factor beta (TGFB), Interferon gamma-inducible protein 16 (*IFI16*) and Interleukin 21 (*IL21*) was significantly higher ($P<0.05$) in heifers from which a viable embryo was recovered compared with those yielding a retarded embryo.

Discussion

Using a unique model of retarded embryo development, this study has established a gene expression profile in the endometrium of cytokines and their transcriptional regulators that may contribute to, or be reflective of, uterine dysfunction. Although limited, there are suggestions in the literature that uncontrolled immune gene activation may perturb the maternal-embryonic dialogue contributing to embryo retardation and ultimately embryo mortality (Hansen *et al.*, 2004). In a previous study (Beltman *et al.*, 2010) we showed that there were no differences in progesterone, IGF-1, insulin and urea concentrations between the 2 groups of heifers and endometrial gene expression pointed towards a dys-regulation in genes involved the TAG and PGF2 α pathway as well as 2 genes involved in the immune response as a potential contributing factor to this phenomenon.

In the endometrium of heifers with a viable embryo, expression of genes involved in the regulation of the immune response were significantly decreased together with the expression of inflammatory cytokines, type I and II interferons and one of the defensins. Hansen *et al* (2004) proposed that the activation of inflammatory cytokines can harm the embryo both directly and indirectly. The expression of *IFNA*, *IFNG* and *IL6*, *IL10*, *FOXP3* and *NCRI* was lower in the endometria from heifers from which a viable embryo was recovered. *IFNA* and *IFNG* are both involved in the Th1 type response i.e. these induce a pro-inflammatory type of response (Lin *et al.*, 1993). *IL6* and *IL10*, which are also pro-inflammatory cytokines, were also decreased in a similar pattern. This pattern was also seen in the expression of *FOXP3* and *NCRI*. Both *FOXP3* and *NCRI* have a regulatory function in the type of immune response, with *FOXP3* is responsible for the major immunological features of regulatory T cells and as such is responsible for the major immunological features of these cells, including immune suppression of conventional T

cells and resistance to Th2 cell differentiation (Zeng et al., 2011). *NCRI* is the natural killer (NK) cell activating receptor in the uterus. NK cells are large lymphocytes that belong to the innate immune system and may provide a link between the two types of immune responses i.e. pro- and anti-inflammatory response. NK cells can produce IFNG when stimulated thus providing a cytokine environment that can induce a Th1 adapted immune response (Maley *et al.*, 2006). The fact that there was lower expression of this gene in the heifers from which a viable embryo was recovered suggests that the pro-inflammatory component of the uterine immune system is less active in these animals. This, together with the decreased expression of the other five genes indicates that in an endometrium from which an appropriately developed embryo is recovered the pro-inflammatory response, while still initiated, is significantly lower than that from which a retarded embryo is recovered.

DEFB1 is one of the antimicrobial peptides which are produced by the neutrophils and epithelial cells and are thought to have a role in the clearance of infection via their immune regulatory capacity (Chapwanya *et al.*, 2009). The decreased expression of this gene in the heifers with a viable embryo heifers suggests that these embryos have a capacity to suppress the localised immune response and as such have an increased chance of surviving in the tract.

The remaining 4 genes had significantly higher expression in the endometrium of heifers yielding a viable embryo and included cytokines and transcriptional regulators (*NKFB1*, *TGFB*, *IFI16* and *IL21*). This suggests that increased expression of these genes may positively benefit the survival of the embryo to blastocyst stage. *TGFB* regulates whether

an immune response will be pro- or anti-inflammatory and as such can play an important role in the response of the endometrium with regards to favourable or not so favourable environment for the developing embryo. High expressions of *TGFB* favours increased *FOXP3* expression, whereas low concentrations are associated with increased *IL6* expression (Zhou *et al.*, 2008), both of which were higher in heifers with retarded embryos whereas the expression of this gene was higher in heifers with viable embryos. An explanation for this could be that the switch between the 2 types of responses (pro- or anti-inflammatory) has already been made in heifers with viable embryos, but that this is not yet the case in heifers with retarded embryos.

In conclusion: it is difficult to differentiate between cause and effect when investigating uterine gene expression differences in the two groups of heifers in this study. In other words, are the less developed embryos leading to the gene expression effects seen in the endometrium or are they a consequence of these differences? Evidence for an effect of the conceptus on the endometrium is provided by two recent elegant studies (Mansouri-Attia *et al* 2009; Bauersachs *et al.*, 2009) both of which show that the type of embryo present on Day 18-20 (cloned, IVF, in vivo derived) can elicit a very different response from the endometrial transcriptome which is reflective of the subsequent developmental outcome. However, there is little if any evidence in the literature to show embryo-induced effects in the endometrium as early as Day 7; indeed, we (Forde *et al.*, 2011) and others (Bauersachs *et al.*, 2012) have failed to detect differences in global transcriptome profile of the endometrium in the presence of a conceptus before Day 13-16. This would strengthen the case for the effect being endometrial rather than embryo in origin.

Our finding of the expression of genes involved in the regulation of the immune response were decreased in the endometrium of heifers from which a viable embryo was recovered also gives weight to the hypothesis of Hansen (2004) and our own hypothesis that the

regulation of the uterine immune response is precise and that subtle changes can change the outcome of the developing embryo.

Given that some of the genes found to be differentially expressed between the 2 groups can regulate whether an immune response will be pro- or anti-inflammatory, the reduced expression of these in endometria from which retarded embryos are recovered indicates that disturbance of the very fine balance between the two responses at this stage of embryonic development can have detrimental implications for embryonic survival

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Figure 1a-d: Calibrated, normalised, relative expression values (CNRQ) in arbitrary units (mean±SEM) for (a) NFκB, (b) TGFβ, (c) IFI16 (d) IL21 with significantly higher expression in the endometrium of heifers from which a viable embryo (n=32) was recovered than in heifers with a retarded embryo (n=19). Mean expression values for normalised, calibrated relative expression are given in arbitrary units. An asterix (*) depicts significant difference (P<0.05) between the 2 groups of heifers. SEM is displayed in the error bars.

Figure 2a-g: Calibrated, normalised, relative expression values (CNRQ) in arbitrary units (mean±SEM) for (a) DEFB1, (b) IFNA, (c) IFNG (d) IL6 (e) FOXP3 (f) IL10 and (g) NCR1 with significantly higher expression in the endometrium of heifers from which a viable embryo (n=32) was recovered than in heifers with a retarded embryo (n=19).. Mean expression values for normalised, calibrated relative expression are given in arbitrary units. An asterix (*) depicts significant difference (P<0.05) between the 2 groups of heifers. SEM is displayed in the error bars.