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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.
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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 defension 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 (NCR1) 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 $(TGF\beta)$, Interferon gamma-inducible protein 16 (IFI16) and Interleukin 21 (IL21) was higher in viable than in retarded heifers. We propose that small disturbances in the 28 29 expression of immune genes in the endometrium on Day 7 after estrus can have 30 detrimental effects on embryonic survival.

31 Introduction

32 In a previous study (Beltman et al., 2010) the relationship between systemic (endocrine/ 33 physiological) and uterine (endometrial gene expression) dysfunction in the initial period 34 of embryonic development from fertilisation to blastocyst formation in subfertile beef 35 heifers was investigated. Two genes, Lysozyme 2 (LYZ2) and Ubiquitin-like with PHD 36 and ring finger domains 1 (UHRF1) that are closely associated with the regulation of the 37 immune system were increased in expression in the endometrium of heifers that yielded 38 retarded embryos on Day 7. Tight regulation of the immune system is required as 39 increased inflammatory cytokines disrupt the hypothalamic-pituitary-gonad axis (Hansen 40 et al., 2004). At a local level, regulation of the immune system in the endometrium is 41 already evident as early as day 7 of pregnancy (Low et al., 1990) and is critically 42 important in pregnancy recognition as well as facilitating implantation (Forde et al., 2010; 43 Mansouri-Attia et al., 2009; Walker et al., 2010; Bauersachs et al., 2012). Because embryo 44 development is dependent on a tight regulation of the maternal immune system (Hansen, 45 1997; Leung et al., 2000; Hansen, 2011), expression of components of the immune system 46 in the endometrium could be associated of successful or unsuccessful embryonic 47 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 48 49 degenerate embryos.

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51 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,

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

56

57 Animal management and treatments

58 The experimental design used for this study was as previously described (Beltman et al., 59 2010). Oestrus was synchronised in cross-bred beef heifers (n=157) using a Controlled 60 Intravaginal Drug Releasing device (CIDR) protocol. Heifers detected in standing estrus 61 (within 24-48 h post CIDR removal, n=102) were inseminated (o estrus=Day 0) with 62 frozen-thawed semen from a single ejaculate of a bull of proven fertility. Tissue collection 63 took place at slaughter on Day 7 post-oestrus. Heifers from which an embryonic structure 64 was recovered were classified as either (i) viable, when the embryo was at the correct 65 developmental stage (i.e. morula/early blastocyst), or (ii) retarded, when the embryo was 66 arrested at the 2- to 16-cell stage. Heifers from which an unfertilised oocyte was recovered 67 or from which no structure was recovered were omitted from the study. Strips of 68 endometrial tissue from the uterine horn were processed stored at -80°C prior to RNA 69 extraction.

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71 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 downregulation 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 byBeltman *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 RNaseand DNase-free water.

85 All reactions were carried out in duplicate and cycling conditions were 50°C for 2 min, 86 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min and were carried out 87 with the inclusion of a dissociation curve to ensure specificity of amplification. A standard 88 curve was included for each gene to generate arbitrary expression values for all genes 89 examined. Qbase plus software was used to perform a geNorm study to determine the most 90 appropriate reference gene for our model system (Vandesompele *et al.*, 2002). The optimal 91 number of reference targets in this experimental situation was determined as 3 (geNorm V 92 < 0.15 when comparing a normalisation factor based on the 3 or 4 most stable targets). As 93 such, the optimal normalisation factor was calculated as the geometric mean of reference 94 targets ACTB, RPL19, and PPIA. All expression data for genes of interest are expressed as 95 mean calibrated normalised relative expression values in arbitrary units (CNRQ values). 96 Significant differences in gene expression between groups were determined by a Students

97 t-test (Snedecor and Cochran, 1989) when the *P* value was < 0.05.

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101 **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.

108 The results of the qRT-PCR analysis are displayed in Figure 1 and 2. Eleven genes were 109 significantly differentially expressed in the endometrium of heifers yielding viable 110 compared with retarded embryos. Of these 11 genes, the expression of Beta defensin 1 111 (DEFB1), Interferon alpha (IFNA), Interferon gamma (IFNG), Interleukin 6 (IL6), 112 Interleukin 10 (IL10), Forkhead box P3 (FOXP3) and Natural cytotoxicity triggering 113 receptor 1 (NCR1) was significantly lower (P < 0.05) in the endometrium of heifers from 114 which a viable embryo was recovered compared with those yielding a retarded embryo. 115 In contrast, endometrial expression of Nuclear factor of kappa light polypeptide gene 116 enhancer in B-cells 1 (NKFB1), Transforming growth factor beta (TGFB), Interferon gamma-inducible protein 16 (IFI16) and Interleukin 21 (IL21) was significantly higher 117 118 (P < 0.05) in heifers from which a viable embryo was recovered compared with those 119 yielding a retarded embryo.

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121

123 **Discussion**

124 Using a unique model of retarded embryo development, this study has established a gene 125 expression profile in the endometrium of cytokines and their transcriptional regulators that 126 may contribute to, or be reflective of, uterine dysfunction. Although limited, there are 127 suggestions in the literature that uncontrolled immune gene activation may perturb the 128 maternal-embryonic dialogue contributing to embryo retardation and ultimately embryo 129 mortality (Hansen et al., 2004). In a previous study (Beltman et al., 2010) we showed that 130 there were no differences in progesterone, IGF-1, insulin and urea concentrations between 131 the 2 groups of heifers and endometrial gene expression pointed towards a dys-regulation 132 in genes involved the TAG and PGF2 α pathway as well as 2 genes involved in the 133 immune response as a potential contributing factor to this phenomenon.

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135 In the endometrium of heifers with a viable embryo, expression of genes involved in the 136 regulation of the immune response were significantly decreased together with the 137 expression of inflammatory cytokines, type 1 and II interferons and one of the defensions. 138 Hansen et al (2004) proposed that the activation of inflammatory cytokines can harm the 139 embryo both directly and indirectly. The expression of IFNA, IFNG and IL6, IL10, FOXP3 140 and NCR1 was lower in the endometria from heifers from which a viable embryo was 141 recovered. IFNA and IFNG are both involved in the Th1 type response i.e. these induce a 142 pro-inflammatory type of response (Lin et al., 1993). IL6 and IL10, which are also pro-143 inflammatory cytokines, were also decreased in a similar pattern. This pattern was also 144 seen in the expression of FOXP3 and NCR1. Both FOXP3 and NCR1 have a regulatory 145 function in the type of immune response, with FOXP3 is responsible for the major 146 immunological features of regulatory T cells and as such is responsible for the major 147 immunological features of these cells, including immune suppression of conventional T

148 cells and resistance to Th2 cell differentiation (Zeng et al., 2011). NCR1 is the natural 149 killer (NK) cell activating receptor in the uterus. NK cells are large lymphocytes that 150 belong to the innate immune system and may provide a link between the two types of 151 immune responses i.e. pro- and anti-inflammatory response. NK cells can produce IFNG 152 when stimulated thus providing a cytokine environment that can induce a Th1 adapted 153 immune response (Maley et al., 2006). The fact that there was lower expression of this 154 gene in the heifers from which a viable embryo was recovered suggests that the pro-155 inflammatory component of the uterine immune system is less active in these animals. 156 This, together with the decreased expression of the other five genes indicates that in an 157 endometrium from which an appropriately developed embryo is recovered the pro-158 inflammatory response, while still initiated, is significantly lower than that from which a 159 retarded embryo is recovered.

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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.

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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 172 an immune response will be pro- or anti-inflammatory and as such can play an important 173 role in the response of the endometrium with regards to favourable or not so favourable 174 environment for the developing embryo. High expressions of TGFB favours increased 175 FOXP3 expression, whereas low concentrations are associated with increased IL6 176 expression (Zhou et al., 2008), both of which were higher in heifers with retarded embryos 177 whereas the expression of this gene was higher in heifers with viable embryos. An 178 explanation for this could be that the switch between the 2 types of responses (pro- or anti-179 inflammatory) has already been made in heifers with viable embryos, but that this is not 180 yet the case in heifers with retarded embryos.

181 In conclusion: it is difficult to differentiate between cause and effect when investigating 182 uterine gene expression differences in the two groups of heifers in this study. In other 183 words, are the less developed embryos leading to the gene expression effects seen in the 184 endometrium or are they a consequence of these differences? Evidence for an effect of the 185 conceptus on the endometrium is provided by two recent elegant studies (Mansouri-Attia 186 et al 2009; Bauersachs et al., 2009) both of which show that the type of embryo present on 187 Day 18-20 (cloned, IVF, in vivo derived) can elicit a very different response from the 188 endometrial transcriptome which is reflective of the subsequent developmental outcome. 189 However, there is little if any evidence in the literature to show embryo-induced effects in 190 the endometrium as early as Day 7; indeed, we (Forde et al., 2011) and others (Bauersachs 191 et al., 2012) have failed to detect differences in global transcriptome profile of the 192 endometrium in the presence of a conceptus before Day 13-16. This would strengthen the 193 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 changethe 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 arbitraty units (mean \pm SEM) for (a) NFKB, (b) TGFB, (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.

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Figure 2a-g: Calibrated, normalised, relative expression values (CNRQ) in arbitraty units (mean \pm 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.

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