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Title	Altered endometrial immune gene expression in beef heifers with retarded embryos
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Publication date	2012-10
Publication information	Beltman, Marijke Eileen, Niamh Forde, Patrick Lonergan, and Mark Crowe. "Altered Endometrial Immune Gene Expression in Beef Heifers with Retarded Embryos." CSIRO Publishing, October 2012. https://doi.org/10.1071/RD12232.
Publisher	CSIRO Publishing
Item record/more information	http://hdl.handle.net/10197/4665
Publisher's version (DOI)	10.1071/RD12232

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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.

15

Abstract

The objective was to compare endometrial gene expression profiles in a group of beef heifers yielding viable or degenerate embryos on Day 7 after oestrus as a means to potentially explain differences in embryo survival rates. The focus was on genes that were associated with either the pro- or anti-inflammatory immune response. Endometrial gene expression was determined using q-RT-PCR analysis. Expression of Beta defensin (*DEFB*), Interferon alpha (*IFN*α), Interferon gamma (*IFN*γ), Interleukin 6 (*IL6*), Interleukin 10 (*IL10*), Forkhead box P3 (*FOXP3*) and Natural cytotoxicity triggering receptor 1 (*NCR1*) was lower in endometria from heifers that produced viable embryos compared with those that produced retarded embryos. Expression of Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NKFB1*), Transforming growth factor beta (*TGF*β), 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 expression of immune genes in the endometrium on Day 7 after estrus can have 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
48 broad range of immune-related genes in the endometrium of heifers producing viable and
49 degenerate embryos.

50

51 **Materials and Methods**

52 All experimental procedures involving animals were approved by the University's Animal
53 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.

70

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

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

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

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

98

99

100

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.

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 PGF2α pathway as well as 2 genes involved in the
133 immune response as a potential contributing factor to this phenomenon.

134

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 defensins.
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.

160

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

167

168 The remaining 4 genes had significantly higher expression in the endometrium of heifers
169 yielding a viable embryo and included cytokines and transcriptional regulators (*NKFB1*,
170 *TGFB, IFI16* and IL21). This suggests that increased expression of these genes may
171 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.

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

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

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

204

205 **Acknowledgements**

206 This work was supported by Science Foundation Ireland Strategic Research Cluster grant
207 code 07/SRC/B1156 (the opinions, findings and conclusions or recommendations
208 expressed in this material are those of the authors and do not necessarily reflect the views
209 of Science Foundation Ireland) and the research fund from the School of Agriculture, Food
210 Science and Veterinary Medicine, University College Dublin, Ireland. The authors thank
211 Dr. John Browne for help with the transcriptomic analyses, the IAD Castlebellingham for
212 access to experimental animals and Dr. Kieran Meade for the critical reading of the
213 manuscript and the advice during the writing process. Finally, the authors like to thank
214 Pfizer Animal Health (Sandwich, UK) for providing the CIDRs for this study.

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293 Figure 1a-d: Calibrated, normalised, relative expression values (CNRQ) in arbitraty units
294 (mean±SEM) for (a) NFKB, (b) TGFB, (c) IFI16 (d) IL21 with significantly higher
295 expression in the endometrium of heifers from which a viable embryo (n=32) was recovered
296 than in heifers with a retarded embryo (n=19). Mean expression values for normalised,
297 calibrated relative expression are given in arbitrary units. An asterix (*) depicts significant
298 difference (P<0.05) between the 2 groups of heifers. SEM is displayed in the error bars.
299
300

301 Figure 2a-g: Calibrated, normalised, relative expression values (CNRQ) in arbitraty units
302 (mean±SEM) for (a) DEFB1, (b) IFNA, (c) IFNG (d) IL6 (e) FOXP3 (f) IL10 and (g) NCR1
303 with significantly higher expression in the endometrium of heifers from which a viable
304 embryo (n=32) was recovered than in heifers with a retarded embryo (n=19).. Mean
305 expression values for normalised, calibrated relative expression are given in arbitrary units.
306 An asterix (*) depicts significant difference (P<0.05) between the 2 groups of heifers. SEM is
307 displayed in the error bars.
308
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310