

Frank H. Rowson evidence
Appendix 2

Short Communication

Bovine fetal microchimerism in normal and embryo transfer pregnancies and its implications for biotechnology applications in cattle*Lauretta Turin*^{1,*,**}, *Pietro Invernizzi*^{2*}, *Marion Woodcock*⁴, *Francesca R. Grati*^{2,3}, *Federica Riva*¹, *Giulia Tribbioli*¹ and *Götz Laible*⁴¹Dipartimento di Patologia Animale, Igiene e Sanita' Pubblica Veterinaria, Universita' degli Studi di Milano, Milano, Italy²Dipartimento di Medicina, Chirurgia e Odontoiatria (Polo San Paolo), Universita' degli Studi di Milano, Milano, Italy³TOMA Advanced Biomedical Assays S.p.A., Busto Arsizio, Italy⁴AgResearch, Ruakura Research Centre, Hamilton, New Zealand

Fetal cells and DNA have been detected in the maternal circulation during and after pregnancy in a few mammalian species. The incidence of similar microchimerism in cattle could have repercussions for the application of modern biotechnologies such as the transfer of transgenic embryos. To determine if fetomaternal leakage can occur in pregnant cows, we have analyzed maternal blood samples for the presence of fetal DNA during gestation and post-partum periods. Y chromosome-specific DNA was detected in up to 73% of blood samples from naturally mated heifers carrying conventional bull calves and a transgene-specific sequence in up to 50% of recipient cows carrying transgenic fetuses. These findings document for the first time that transplacental leakage of fetal DNA into the maternal circulation can occur in cattle despite the epitheliochorial placenta of ruminants, with potential implications for the utilization of recipient cows in the food chain.

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The presence within one individual of a small population of cells or DNA from another genetically distinct individual is referred to as microchimerism. The phenomenon is well documented in humans resulting from the bi-directional exchange of cells between fetus and mother during pregnancy [1]. In women, fetal DNA has been consistently found in the maternal circulation from early pregnancy to years after delivery [2–4]. The presence of circulating fetal DNA or cells has practical implications in human medicine for non-invasive prenatal diagnoses based on maternal blood, and has been linked to pathophysiological

effects in some autoimmune disorders such as systemic sclerosis and Hashimoto's thyroiditis [5–8]. The potential occurrence of microchimerism in cattle has to be considered in a different context as they play a key role in the human food supply. Due to their importance for agriculture, they are an obvious choice for the application of new biotechnologies such as transgenesis aimed at the utilization and improvement of their production characteristics [9]. It is, therefore, not surprising that transgenic (Tg) cattle lines with improved production characteristics, disease resistance and for biopharming applications have been described in recent years [10–13]. In many cases, these biotechnology applications involve the production of embryos *in vitro* followed by their transfer into synchronized recipient females for development to term.

Correspondence: Dr. Götz Laible, AgResearch Ruakura Research Centre, Hamilton, New Zealand**E-mail:** goetz.laible@agresearch.co.nz**Fax:** +64 7 838 56 28**Abbreviations:** HBB, beta-globin gene; mtDNA, mitochondrial DNA; NT, nuclear transfer; NTC, no-template control; Tg, transgene; Y-S4, Y-chromosome-specific repeat sequence

*These authors equally contributed to this work.

**Additional corresponding author: Dr. Lauretta Turin
E-mail: lauretta.turin@unimi.it

Transplacental leakage of cells derived from such a 'manipulated' fetus, in particular if the fetus is Tg, would have immediate implications for the suitability of the recipient cow to enter the human food chain, especially if these cells have the ability to persist and engraft into maternal tissues. In our studies, all recipients of Tg embryos are under strict regulatory controls and are treated as Tg animals. They have to be kept in a physical containment facility and cannot enter the human food chain. Although not a major issue at the small scale of a research project, restrictions for recipients when projected onto a larger scale after the commercial uptake of such technologies would translate into a significant financial hurdle.

In humans, the fetomaternal transfer of cells is made possible through the direct contact of the maternal blood system with the fetal epithelial membrane layer characteristic for the highly invasive hemochorial placenta of primates and rodents [5, 14]. In comparison, cows have a less invasive epitheliochorial placenta [15], which features closed maternal and fetal vasculature systems and six tissue layers separating fetal and maternal blood [16–18]. Thus, the bovine placenta presents a much greater barrier and should be less prone if not restrictive to the high incidence of transplacental cell leakage seen in humans.

Rather unexpectedly, microchimerism in cattle has been previously detected as the result of maternal-fetal transplacental leakage in a study investigating potential mitochondrial DNA (mtDNA) heteroplasmy in cloned bovine fetuses [19]. This indicated that at least in nuclear transfer (NT) pregnancies, which often suffer from aberrant placentation [20–22], the placental barrier can be compromised and allow transplacental passage of cells from the mother to the fetus. A subsequent report confirmed this finding and described maternal-fetal transplacental leakage in two out of eight nuclear transfer pregnancies [23]. Moreover, maternal mtDNA was also found in one out of seven pregnancies with embryos generated by *in vitro* fertilization. Microchimerism could not be detected in pregnancies established after transfer of *in vivo* produced embryos, although only a total of four fetuses were analyzed. Thus, it has been argued that placental anomalies, also observed at a lower frequency with *in vitro* produced embryos, together with the additional strain on the reproductive system through *in vitro* techniques might be a major factor associated with the disruption of the placental barrier and allow placental transfer of both fetal and maternal cells [23].

Despite its relevance for Tg applications, cell transfer in the fetomaternal direction has never been investigated in cattle. To evaluate the possibility of DNA leakage from the fetus to the mother, we have analyzed maternal blood samples from pregnant cows for fetal-derived DNA sequences using sensitive real-time PCR assays. The study was based on two groups of animals, naturally mated heifers and conventional recipient cows pregnant after

non-surgical transfer of a single Tg embryo produced by *in vitro* fertilization of transvaginally recovered oocytes [24] from TG3 cows [10]. While the heifers were allowed to calve naturally, parturition was induced in the recipient cows about 2 weeks before the expected full-term calving date as previously described [25]. Heifers with male pregnancies ($n=16$) were analyzed at mid-gestation (around day 150 of gestation), pre-calving (around days 260 of gestation), at calving (within 2 days after calving) and 4 months post-calving for the presence of the Y chromosome-specific repeated sequence S4 (Y-S4) [26] indicative of transplacental leakage from the male fetus to the mother. Similarly, pregnant recipient cows of Tg embryos ($n=10$) were tested at the same four stages for the presence of a chimeric β/κ -casein (CSN2/3) transgene sequence, four copies of which are part of the genome of the transferred Tg fetuses but which is absent in conventional recipient cows [10]. In addition, recipients of Tg embryos were also analyzed 25–26 months after the delivery of their Tg calves to investigate long-term persistence of microchimerism. Whereas all 16 enrolled heifers were at their first pregnancy, recipients of Tg embryos were cows that had at least born and successfully reared a conventional calf before entering the program. Some of the recipient cows received a transfer of a Tg CSN2/3 embryo 18–30 months before the trial. While most of these transfers failed to establish a pregnancy beyond the early stages, one of the recipients had given birth to a single Tg calf prior to this study. In addition, the study included cows that have never been pregnant, mothers of female offspring and mothers of non-Tg offspring as negative controls and samples from bulls and Tg cows as positive controls.

To investigate the presence of the Y-S4 sequence or the chimeric CSN2/3 sequence [10] in the maternal circulation, genomic DNA was extracted from peripheral blood samples using the Qiagen Blood Kit (Qiagen, Chatsworth, CA, USA). For each sample, three independent DNA extractions were performed starting from 500 μ L whole blood, which were subsequently tested using a real-time quantitative PCR (Taqman) assay. The quantification of the target sequences was normalized to the endogenous single copy β -globin gene (HBB). All primers and probes (Y-S4-forward 5'-GGAAACTGTCATTCAAGCTGCATAG-3', Y-S4-reverse 5'-ACACACACACAGTTACAGTGTGTTGT-3', Y-S4-probe 5'-CACAGCGAAAATATG-3'; Tg-forward 5'-TGAGAGCCATGAAGGTCCTCA-3', Tg-reverse 5'-CTTGGTTTTGCTCCTGTGCA-3', Tg-probe 5'-CTGCCTGG-TGGCTCTGGCC-3'; HBB-forward 5'-TGCAGGCTGACTTTCAGAAGG-3', HBB-reverse 5'-AGCTTAATGATATCTGTGGGCCA-3', and HBB-probe 5'-TGGCTGGTGTGGCCAATGCC-3') were designed using the Primer Express Software 2.0 (Applied Biosystems, Foster City, CA, USA) according to sequences retrieved from the GenBank Sequence Database (accession nos. gi:51036014 for Y-S4, gi:394 for HBB, gi:459291 for Tg-forward and Tg-

probe). Tg-reverse spans the junction between the CSN2 (gi:459291) and CSN3 (gi:170) genes in the chimeric CSN2/3 construct. PCR was performed using the ABI Prism Amplification 7000 Sequence Detector System (Applied Biosystems) essentially as described previously [27]. For each sample, at least two of the three independently extracted DNAs were analyzed. Each experiment was carried out in duplicate, with a calibration curve and no-template control (NTC) being run in parallel and in duplicate with each analysis. In particular, tenfold serial dilutions of quantified DNAs from a wild-type male and Tg cow were used to generate standard curves for estimation of the copy number for each sequence investigated. A blood sample was scored as positive if one or more of the four to six tested replicates showed amplification of the target sequence. Moreover, extensive precautionary measures were taken to exclude the risk of PCR product carry-over and false-positive amplification including the spatial separation and use of dedicated laminar flow cabinets for the different processing steps [3], pre-amplification treatment with uracil N-glycosylase [28] and the handling and testing of all samples by female operators only.

Our real-time PCR test and procedures proved to be highly sensitive, with comparable sensitivities for both assays, yet very reliable for the detection of the target DNA. In the absence of any false-positive PCR results with all negative control animals ($n=9$ for Y; $n=9$ for Tg) and NTC samples ($n=54$) showing no target amplification, we were able to classify even a single positive PCR reaction as an indicator for a blood sample containing fetal-derived DNA sequences. The fetal-derived male-specific Y-S4 sequence could be detected in some of the conventional heifers at all time points tested (Fig. 1a). Blood samples obtained at mid-gestation (25%), at pre-calving (40%), at calving (73%) and 40% of blood samples from 4 months post-calving contained fetal-derived Y-S4 DNA sequences. The transgene-specific CSN2/3 sequence was undetectable in mid-gestation blood samples but was readily detectable in samples obtained at later stages (Fig. 1b). CSN2/3 sequences were present in maternal blood samples obtained at pre-calving (20%), at calving (11%) and 4 months post-calving (50%). In samples taken more than 2 years after calving, the CSN2/3 sequence was detected in a single animal (10%).

When we further analyzed the occurrence of positive samples over the investigated period, we observed a statistically significant peak of positive events for the Y-S4 sequence at calving ($p=0.039$, exact binominal test for increase from mid-gestation to calving) where 11 of 12 (92%) positive cows showed detectable levels of the male-specific sequence (Table 1). A comparison of all PCR tests (Y-S4 $n=84$; CSN2/3 $n=80$) for the two study groups, using Two-Sample *t*-test, revealed that the proportion of cows with detectable microchimerism was significantly higher for the Y chromosome-specific Y-S4 sequence than with the transgene-specific CSN2/3 sequence at calving ($p=1$

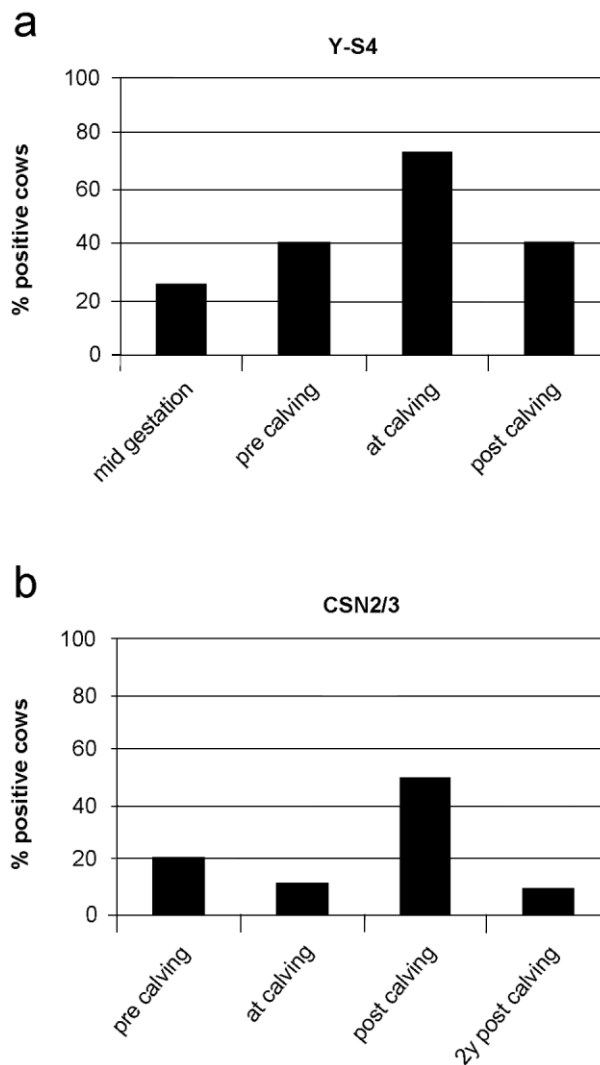


Figure 1. Percentage of cows carrying fetal-derived sequences. (a) Percentage of blood samples from naturally mated heifers in their first pregnancy positive for the presence of the male-specific Y-S4 sequence, and (b) percentage of samples from conventional recipient cows pregnant with *in vitro* produced Tg embryos positive for the chimeric β/κ -casein (CSN2/3) transgene-specific sequence. Microchimerism was analyzed by real-time PCR at different gestational stages as indicated. (b) The 2-year post-calving results only determined for the recipient of the Tg embryos are included. The mid-gestation data were all negative and have been omitted. Blood samples were considered positive if at least one of the replicates gave a positive result.

$\times 10^{-7}$). The Tg study group showed the highest incidence of microchimerism (50%) at post calving ($p=0.125$, exact binominal test for increase from mid-gestation to calving) (Table 2). The shift of the peak in the Tg group from calving to post calving appears to be a reflection of differences in the time of sampling at calving. The blood samples in the Tg group were collected 1 h after calving. Any fetal material that might have entered the maternal circulation during parturition could have been still concentrated lo-

Table 1. Presence of fetal DNA in maternal blood during different stages of pregnancy in cows with microchimerism in naturally mated heifers analyzed for the presence of fetal derived Y-S4 DNA^{a)}

Stage of pregnancy	Cows											
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
Mid-gestation	–	–	–	–	–	–	+	–	+	–	+	+
Pre calving	–	–	–	–	–	+	–	+	+	+	+	+
At calving ^{b)}	+	+	+	+	+	+	+	+	–	+	+	+
Post-calving	–	–	–	–	–	–	+	+	+	+	+	+

a) +: Positive identification of the target DNA in at least one of six replicates; –: negative for the target DNA in all six replicates; ND: not determined. Note, the table excludes all animals that did not contain fetal DNA in any blood sample.

b) 'At calving' represents 2 days postpartum.

cally, preventing the detection in blood samples taken from the jugular vein. Sampling within an hour was impossible for the heifers that were part of a much larger herd and these were sampled within 2 days of calving. In part, the observed difference might also be attributable to the increased strain associated with the first delivery of a calf by heifers in comparison to multiparous cows.

Overall, our results provide evidence for bovine fetomaternal microchimerism not only during pregnancy, but also in the postpartum period, as has been described in humans [29]. Yet, there appears to be a marked difference in the frequency of fetomaternal microchimerism during pregnancy and postpartum. For both study groups, only about one third of positive samples occurred within the gestation period (Tables 1 and 2). This appears to indicate that the incidence of microchimerism in cattle can originate at a low frequency from transplacental leakage during gestation, whereas the predominant transfer of fetal-derived genetic material is related to parturition, which might present a brief window of opportunity for the exchange of fetal and maternal blood. Irrespective of when the fetal-derived DNA sequences are detected, during pregnancy or postpartum, in many instances the detected fetal DNA is present at one point but absent in the subsequent sample (Table 1), which indicates that the observed microchimerism might be only transient. Blood samples taken 2 years after the delivery of a Tg calf confirmed the predominantly transient nature of circulating

fetal DNA sequences. Most of the previously positive cows were clear of the CSN2/3 sequence, which remained detectable in a single animal only (Table 2). Transient microchimerism is also consistent with our observation that all blood samples from one recipient cow that had borne a Tg CSN2/3 calf before this trial scored negative for the presence of fetal sequences.

Although we detected a significantly higher proportion of cows with microchimerism for the Y-S4 sequence than for the transgene-specific CSN2/3 sequence, this was not correlated with a higher absolute amount of fetal DNA. On the contrary, quantification of fetal DNA in the maternal blood, as shown in Fig. 2, revealed a markedly higher passage of the Tg CSN2/3 sequences compared to Y-S4 sequences. Average blood levels for the Y-S4 sequences were around 0.04 ng/mL, whereas CSN2/3 sequence levels were with 0.24 ng/mL about sixfold higher. The detected amount of fetal-derived DNA corresponds to 12 haploid genome equivalents per mL of blood for the male-specific sequence and to 72 genome equivalents per mL for the Tg sequence, respectively. Thus, the magnitude of the microchimerism is in the order of six circulating male cells or their corresponding DNA contents (if cell-free) per mL of maternal blood and about 36 Tg cells, respectively. This is in good agreement with the range of 20–149 genome equivalents/mL that was reported in the literature for Y-specific sequences in women [3].

Table 2. Presence of fetal DNA in maternal blood during different stages of pregnancy in cows with microchimerism in recipients of Tg embryos analyzed for the presence of fetal-specific CSN2/3 DNA^{a)}

Stage of pregnancy	Cows					
	R1	R2	R3	R4	R5	R6
Mid-gestation	–	–	–	–	–	–
Pre calving	+	+	–	–	–	–
At calving ^{b)}	–	–	+	–	–	–
Post-calving	–	–	+	+	+	+
After 2 years	–	ND	–	–	+	–

a) +: Positive identification of the target DNA in at least one of six replicates; –: negative for the target DNA in all six replicates; ND: not determined. Note, the table excludes all animals that did not contain fetal DNA in any blood sample.

b) One hour postpartum.

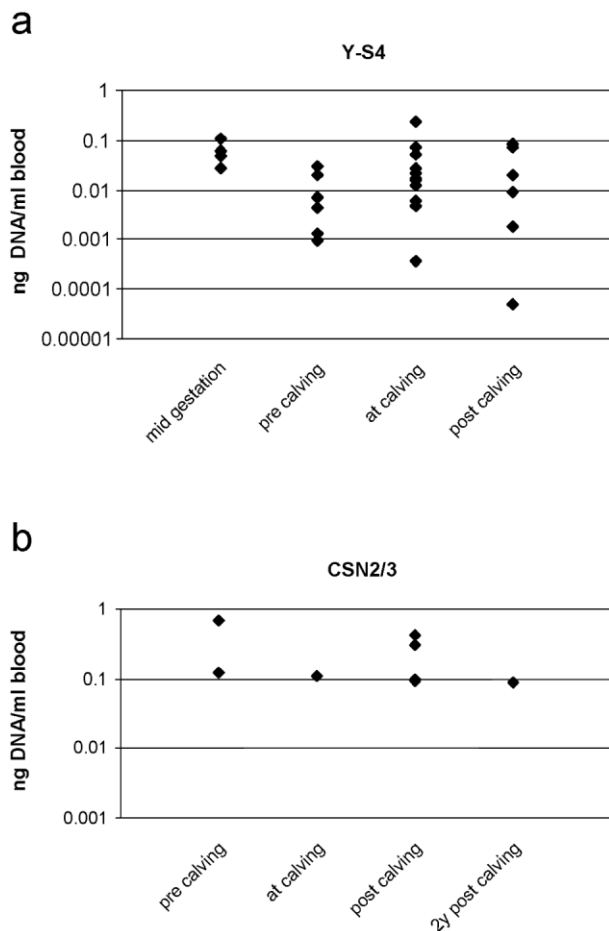


Figure 2. Quantification of the fetal DNA levels in maternal blood. The fetal DNA concentrations are expressed in ng/mL blood on a logarithmic scale versus each time point of the study. Data points represent single quantifications of fetal DNA (mean value of six independent replications) measured in the blood of individual cows with detectable microchimerism for the male-specific Y-S4 sequence (a) and microchimerism for the CSN2/3 transgene sequence (b).

Following the detection of maternal mtDNA in fetuses by Hiendleder and co-workers [23], we could now demonstrate transfer in the opposite direction and were able to detect fetal DNA in the maternal circulation. Thus, the fetomaternal cross-talk in cattle appears to be bi-directional, a characteristic that has been well established for microchimerism observed in humans [1]. Using highly sensitive real-time PCR tools, we detected fetal DNA transfer from mid-gestation in heifers and in cows from pre-calving onwards. The absence of fetal DNA at mid-gestation for the recipient cows is most likely a reflection of a very low occurrence of fetal DNA transfer during early gestation but may also be due to the more limited numbers of animals available for the testing of recipient cows carrying Tg embryos. However, we cannot formally exclude that the lack of any recipient cows with fetal DNA at mid-gestation is due to a genuine difference between

heifers versus cows whereby transplacental leakage develops only at later gestational stages in cows. In both, heifers and cows, the occurrence of microchimerism was highest after parturition. These results agree with that reported in human, where the transfer of fetal cells into the maternal circulation begins at 4–6 weeks of gestational age and continues until parturition with fetal cells persisting even after delivery [30]. Unlike in humans, in cattle this is not correlated with an accumulation of fetal DNA in maternal circulation with progression through gestation. In both study groups, naturally mated heifers and recipients of *in vitro* embryos, the level of fetal DNA found in the maternal circulation up to 4 months post partum remained essentially constant ($p=0.054$ for Y-S4 and $p=0.336$ for CSN2/3, two-way ANOVA test of ranked concentrations between stages) (Fig. 2). At 2 years after calving, the transgene-derived CSN2/3 sequence was no longer detectable in all but one positive recipient. In this particular cow, the 2-year CSN2/3 DNA levels were at the lower end of the range detected in earlier blood samples.

Previously, *in vitro* techniques in conjunction with placental anomalies were suspected to play a key role for microchimerism in cattle [23]. Our study provides evidence that fetal-derived DNA sequences can be present even in naturally mated heifers in the absence of any *in vitro* reproductive technologies. Under *in vivo* conditions placental anomalies should not be a significant factor. Indeed, pregnancy monitoring did not reveal any anomalies, and placental and fetal development proceeded apparently normal in all animals. This suggests that other factors, such as cell-free DNA transfer or placental areas with a less stringent separation of maternal and fetal blood might exist that are implicated in the leakage of the bovine placenta. Moreover, the observed quantitative difference between the Y-S4 and CSN2/3 results suggests that the use of *in vitro* reproductive techniques although not absolutely necessary for microchimerism to occur, could play a role in enhancing transfer across the placenta due to the strain it might impose on the reproductive system. On the other hand, it might also be a reflection of intrinsic differences between a first and repeated pregnancy resulting in increased leakage in multiparous cows compared to heifers pregnant and calving for the first time.

With the recent approval of the first recombinant protein derived from a Tg animal by the European Medicines Agency livestock transgenesis has finally taken the long-awaited step onto the commercial arena [31]. This highlights that the significance of microchimerism in livestock animals needs to be further evaluated. Based on our findings that fetomaternal microchimerism can occur in cattle, it is now important to determine if, similar to humans, fetal sequences (i) may be both associated to nucleated cells and/or be cell-free; and (ii) might engraft into specific maternal tissue sites such as lymphoid organs or bone marrow. This, together with the frequency and per-

sistence of microchimerism incidents in bovine will reveal the significance and potential implications it might hold for new biotechnology applications in cattle.

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