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Quantitation of the mRNA levels of Epo and EpoR in various tissues in the ovine fetus☆

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Abstract

A partial cDNA of the sheep erythropoietin receptor (EpoR) was obtained and used in real-time PCR to quantitate mRNA levels in placenta, liver and kidney throughout development (term = 150 days). This was compared with Epo mRNA levels in the same tissues. Both Epo and EpoR mRNA were present in the placenta throughout gestation at low levels from 66 days onwards and these did not vary throughout gestation. Compared with the expression levels in the placenta, the levels of EpoR gene expression in the liver at 66, 99 and 140 days were, median (range)—288 (120–343), 278 (63–541) and 7 (3–15), respectively, reflecting the disappearance of erythropoiesis after 130 days. Low levels of EpoR gene expression were seen in the kidney at 3 (2–5), 5 (2–7), and 7 (2–10) times that in the placenta at 66, 99, and 140 days, respectively. By hybridization histochemistry the EpoR mRNA was located in the proximal tubular cells of the mesonephros and metanephros at 42 days. Epo mRNA levels in the kidney were 215 (116–867), 528 (113–765) and 46 (15–204) times those in the placenta at 69, 99, and 140 days, respectively. In the liver at the same ages the concentrations of mRNA were lower than in the kidney, the liver/placenta ratios being 50 (11–90), 17 (3–39), 9 (5–14). At 130 days Epo/EpoR levels in the hippocampus were 6 ± 3 and 8 ± 3 times that in the term placenta, respectively. These studies demonstrate that the ovine placenta expresses the Epo gene from at least 66 days of gestation. However, gene expression levels are very low compared with those in the liver and kidney, and even the hippocampus.

Keywords: Ovine; Fetal; Placenta; Erythropoietin; Erythropoietin receptor; Real-time PCR

1. Introduction

Erythropoietin (Epo) is a glycoprotein hormone essential for normal erythropoiesis (Moritz et al., 1997; Tilbrook and Klinken, 1999); it acts to increase viability of the erythroid precursors and stimulates their proliferation and differentiation. The erythropoietin receptor (EpoR) is a single transmembrane protein, two of which must be bound to one Epo molecule to achieve signal transduction (Constantinescu et al., 1999; Wilson and Jolliffe, 1999). The kidney is the major site of production of circulating Epo in the adult, and the cells responsible are the interstitial fibroblast-like cells in the proximity of the proximal tubules (Koury et al., 1989; Lacombe et al., 1988; Darby et al., 1995). Hypoxia is the major stimulus to Epo production and it acts to increase the total amount of Epo mRNA in the kidney, predominantly by increasing the number of cells expressing the Epo gene (Koury et al., 1989; Lacombe et al., 1988; Darby et al., 1995; Fisher et al., 1996).

In the fetus, the liver is a major site of Epo production and is also a major site of erythropoiesis, the erythroid progenitors expressing EpoR (Moritz et al.,

☆ Sequence data from this study has been deposited in the GenBank™/EMBL Data Libraries under the accession numbers AY029231 and AY029232.

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1997; Juul et al., 1998; Pearson et al., 2000). However, in the human fetus, Epo and EpoR have been claimed to be much more widely expressed than the adult (Juul et al., 1998; Dame et al., 2000). Both Epo and EpoR have been claimed to be produced by the human placenta (Conrad et al., 1996; Fairchild Benyo and Conrad, 1999). Whether the placental Epo has a local or systemic effect, or both, is unknown.

With increasing sophistication of molecular biology techniques, it has become possible to detect Epo mRNA in tissues other than the kidney in the adult, such as the brain and uterus (Masuda et al., 1994; Digicaylioglu et al., 1995; Morishita et al., 1996; Marti et al., 1996; Yamaji et al., 1996; Chikuma et al., 2000). In most of these organs there is gene expression of EpoR in a cell/tissue-type relatively close to the cell type expressing the Epo gene suggesting a local paracrine system (Digicaylioglu et al., 1995; Morishita et al., 1996; Yasuda et al., 1993). This suggests Epo may have roles other than the maintenance of adequate erythropoiesis. In the kidney, EpoR is expressed in epithelial cells of the proximal tubule, albeit at low levels (Westenfelder et al., 1999).

In terms of development of erythropoiesis, the sheep fetus has been a widely-used model, as the same type of hemoglobin switching (embryonic to fetal to adult) occurs as in man, and ovine fetuses can be chronically cannulated and blood sampled from mid gestation (Moritz et al., 1997). The liver is the site of erythropoiesis from 26 to 130 days of gestation (term is 145–150 days), after which erythropoiesis begins to occur in the bone marrow. The ovine Epo gene has been cloned (Fu et al., 1993), and found to be expressed in the renal interstitial cells of the kidney in the fetal and adult metanephros (Darby et al., 1995), the transient mesonephros (Wintour et al., 1996), and to be regulated by hemorrhage (Lim et al., 1996). The only major difference between the human and the ovine fetus, with respect to Epo production, is that the ovine kidney and liver appear to contribute about equal amounts from early in gestation (Lim et al., 1994), whereas the liver is the predominant Epo producer for most of human fetal life (Dame et al., 1998). In the ovine fetus the hepatic levels of Epo mRNA are highest at day 60 (term being 145–150 days) whereas the renal Epo mRNA levels are high from 60 to 100 days, decreasing thereafter (Lim et al., 1994).

In the current study we compared the levels of Epo mRNA in the liver and kidneys of ovine fetuses by the first really quantitative approach, real-time polymerase chain reaction (PCR). This is a highly sensitive technique requiring only 5–50 ng of cDNA, and is capable of detecting very low levels of mRNA (Jung et al., 2000; Bustin, 2000). It was thus possible to quantitate the relative levels of placental Epo mRNA also. Preliminary studies were performed on the hippocampus, as another site of relatively low expression of Epo and EpoR. The major hypothesis tested was that Epo production, quantitatively, is much lower in the placenta than in the liver/kidney. In addition, we report the cloning of a partial ovine EpoR cDNA and its quantitation by real-time PCR in the same tissues (kidney, liver and placenta) in fetuses at 66–140 days of gestation. Hybridisation histochemistry was used to determine the cellular localization of EpoR mRNA in the kidney early in the gestation.

2. Methods

2.1. Animals

Authorisation to conduct animal experiments was obtained from the animal Ethics Committee of the Howard Florey Institute. Pregnant Merino ewes of known mating date were killed by an overdose of sodium pentobarbitone (Lethabarb; 100 mg/kg body weight). Tissue samples of placenta, kidney and liver were collected from four fetuses for each group of 66 ± 1; 99 ± 1 and 140 ± 1 days of gestation (term is 150 days). Fresh tissue was snap frozen in liquid nitrogen and stored at -80 °C until use. In addition, placental tissue was obtained from six fetuses at each of 27 and 45 days of gestation, and hippocampus from four fetuses at 130 days of gestation. These animals were used in other protocols in our laboratory. Fixed tissue from fetuses at 42 days of gestation, used in a previous study (Wintour et al., 1996), were used here for hybridisation histochemistry.

2.2. RNA extraction and reverse transcription

Total RNA was isolated from frozen tissue using the acid guanidinium thiocyanate and phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The integrity of the total RNA was examined by electrophoresis of 1 μg on 1% denaturing agarose gels. The samples were stored at -80 °C until further use.

Each total RNA sample was DNAse-treated before reverse transcription. To 70 μl of total RNA a 30 μl aliquot of a DNAse reaction mixture (10 mM DTT, 5 mM $MgCl_2$, 40 mM Tris–HCl [pH 7.5], 20 U RNase inhibitor [Promega], and 3 U DNaseI [Promega]) was added. The sample was then incubated at 37 °C for 15 min, followed by 65 °C for 10 min. For each total RNA sample, 0.1 μg was reverse transcribed in a 10 μl reaction containing $1\times$ TaqMan® RT buffer, 5.5 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM random hexamers, 0.4 U/μl Rnase inhibitor, and 1.25 U/μl MultiScribe™ reverse transcriptase (Applied Biosystems). In order to assure that there was no genomic DNA contamination, controls in which no reverse transcriptase

was added were also carried out. The reverse transcriptions reactions were incubated at 25 °C for 10 min, 48 °C for 30 min, 95 °C for 5 min and then stored at 4 °C. The reverse transcription reactions were carried out in a GeneAmp PCR System 9600 (Applied Biosystems).

2.3. Cloning of the ovine EpoR

In initial experiments, oligonucleotide primers were synthesized based on the published sequence of the mouse EpoR gene (Genbank Accession No. J04843) in regions which shared homology with the human EpoR sequence (M60459). cDNA samples from a 60 days fetal sheep liver were used for amplification. PCR products were electrophoresed and blotted onto nylon membranes (Hybond N^+, Amersham), then probed with P^{32}-labelled internal oligonucleotides. Of several oligonucleotide primer sets used, only one gave a positive result by Southern hybridization. The primers used were located in exons VII and VIII, with the sequence of 5′ CGGAATTCAGAAGATCTGGCCTGGCAT -3′ and 5′-CTAGTCTAGAACCACAAGGTATAGG-TACTT-3′, respectively. Semi-nested PCR, as well as Southern hybridization with an internal sequence, 5′-CTAGTCTAGATGTCCTGGGCATGCTCACT-3′ were carried out to confirm that the band was the sheep EpoR. PCR products were subcloned into the Bluescript KS vector (Stratagene), taking advantage of unique restriction fragment sites that had been incorporated into the 5′ end of the oligonucleotides, and sequenced.

Subsequently, additional cDNA sequence from Exon II to VII was obtained by designing a 5′ sense primer from the sequence in Exon II common to rat, cow and man, 5′-GACTTGGTGTGTTTCTGGGA-3′, and the antisense primer 5′-ATGCCAGGCCAGATCTTCTG-3′ from sequence in exon VII which was identical in rat and man. The PCR products were sequenced directly using the Applied Biosystems BigDye terminator kit (Applied Biosystems). The BLAST (Altschul et al., 1997) and Proalign (Rognes, 2001) programs were used to perform searches for homologous sequences.

2.4. Real-time PCR

Real-time quantitative multiplex PCR of Epo and the endogenous control 18S rRNA (18S), and EpoR and 18S was performed using an ABI PRISM 7700 Sequence Detector (Applied Biosystems), by methods described in detail previously (Johnston et al., 2000; Moritz et al., 2000). Primers and probes for Epo and EpoR were designed using Primer Express™ version 1.0 (Applied Biosystems). For Epo, a 70 bp (nt 137–206) fragment was amplified using the forward primer 5′-CCAGGGAGGCCGAAAATG-3′ (900 nM) and the reverse primer 5′-GGGACAGTGATATTCTCACT-GAAGCT-3′ (50 nM). A TaqMan® probe, 5′- FAM (6-carboxy fluorescein)-CAGCCTTCTGCACAGCCA-TCGT-3′ TAMRA (6-carboxy-tetramethylrhodamine) (Applied Biosystems), was included with the primers in each PCR reaction at 100 nM. Similarly, for EpoR, a 76 bp (nt 964–1039) fragment was amplified using the forward primer 5′-CAGCCTTGTGGCGATATGG-3′ (900 nM) and the reverse primer 5′-GGGCCAAAGC-GGATG-3′ (900 nM), plus a TaqMan® probe, 5′ FAM-CATAGTGACCACGGATGAAGCCTCAGAA-3′ T-AMRA (125 nM). The TaqMan® probe and primers for 18S were supplied by Applied Biosystems in a control reagents kit.

Multiplex PCR reactions were carried out in volumes of 25 μl consisting of 1 × TaqMan® 18S (50 nM) probe, 20 nM 18S forward primer, 80 nM 18S reverse primer, as well as the Epo or EpoR probes and primers. Concentrations of probes and primers were determined by optimization experiments using adult kidney mRNA. Amplification of the cDNA (50 ng) and the no RT controls were carried out using the following conditions: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

The comparative C_T method was used for the calculations of expression of both Epo and EpoR. C_T is the cycle number at which fluorescence first exceeds the threshold, i.e. amplification is first detected. This is determined by the total quantity of the relevant mRNA. The lower the C_T value the greater the amount of mRNA in the sample. Thus the results can be quoted as C_T value or compared with a given calibrator. In our studies the calibrator used was the C_T value for the placenta of the same fetus. The values for 18S C_T were subtracted from Epo or EpoR C_T values of each tube to obtain a ΔC_T value for each sample. Subtraction of the calibrator ΔC_T (ΔC_T for placenta of same fetus) from the sample ΔC_T gave a $\Delta\Delta C_T$ for each sample. As the calibrator has a lower amount of mRNA it has a higher C_T value and the subtraction results in a negative number. Thus a difference of -2 in the $\Delta\Delta C_T$ represents a 4-fold difference in the level of mRNA. The expression of Epo or EpoR in the organ relative to the placenta was then calculated using the expression $2^{-\Delta\Delta C_T}$. For the hippocampus, the value was calculated three times using the ΔC_T for kidney, liver, or placenta of 140 days fetuses. Five aliquots of cDNA from (tissue) were run in one assay and gave an intra-assay coefficient of variation (CV) of 11% for Epo, 15% for EpoR. Individual tissues were repeated twice, but all results quoted are from one assay in which all tissue samples were present.

2.5. Statistics

One way ANOVA, followed by a Tukey test, was

used to determine the significance of levels of Epo and EpoR expression in liver and kidney at various gestational ages. Significance was set at $P < 0.05$.

2.6. Hybridization histochemistry

A cDNA template was generated by PCR, using primers specific to the sheep EpoR sequence and incorporating recognition sites for T7 and SP6 RNA polymerase at the 5′ end (Young et al., 1993). The sequence of the PCR primers were 5′-*CGATTTAGGT GACACTATAG AA*GCGAGCCT TATCCGCTAT GA-3′ for the forward primer, with a T7 RNA polymerase recognition sequence (underlined) followed by sequence complementary to the sheep EpoR exon V, and 5′-*ATTAATACGA CTCACTATAG GG*CTTGTCCA GCATCAGGTA GC-3′ for the reverse primer, which comprised the SP6 RNA polymerase recognition site followed by a sequence complementary to Exon VIII of the sheep EpoR cDNA. The length of the expected PCR product including the RNA polymerase sites was 635 bp. PCR was carried out from sheep bone marrow cDNA. Gel electrophoresis revealed the presence of two bands, the expected band and one of higher molecular weight corresponding to an intron-7 inserted splice variant. DNA from the lower band was isolated and reamplified. The PCR product was then purified using a PCR extraction kit (Qiagen).

Riboprobe generation and hybridisation histochemisty was performed as detailed previously for the Epo gene (Wintour et al., 1996).

3. Results

3.1. Organ weights

Fig. 1 presents the weights of fetus (A), placenta (B), kidney (C) and liver (D) at three stages of gestation. Kidney and liver weights increased with gestational age, as did body weights, whereas the placenta reached maximum weight by mid-gestation.

3.2. Cloning of the ovine EpoR

A 1160 bp partial ovine EpoR cDNA fragment was isolated and sequenced, spanning from Exon II to VIII (Fig. 2). This has been assigned the accession number AY029232. In initial experiments, two bands were obtained with RT-PCR using primers complementary to

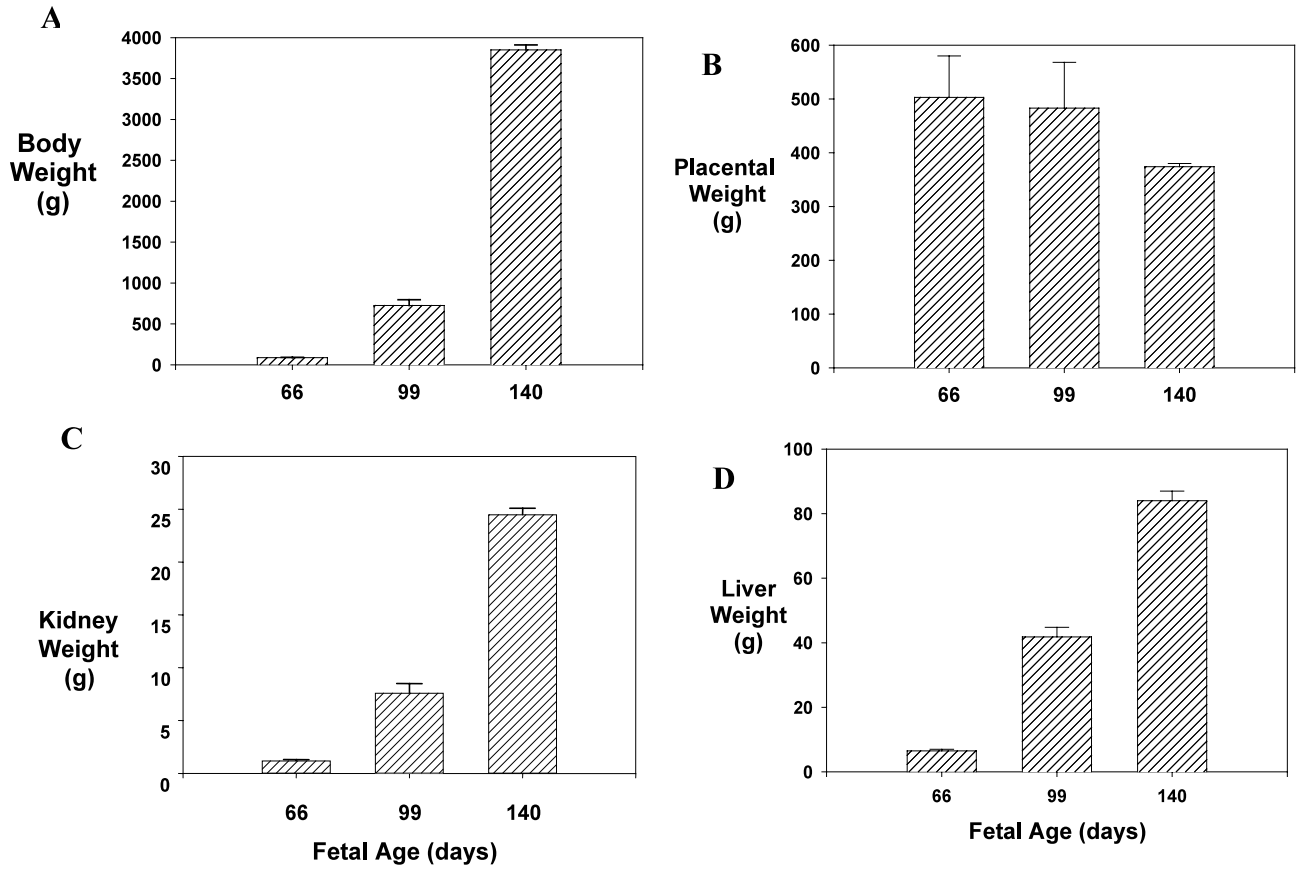

Fig. 1. Weight of fetus (A), placenta (B), kidney (C) and liver (D) at gestational ages 66, 99 and 140 days of gestation. $n = 4$ at each age.

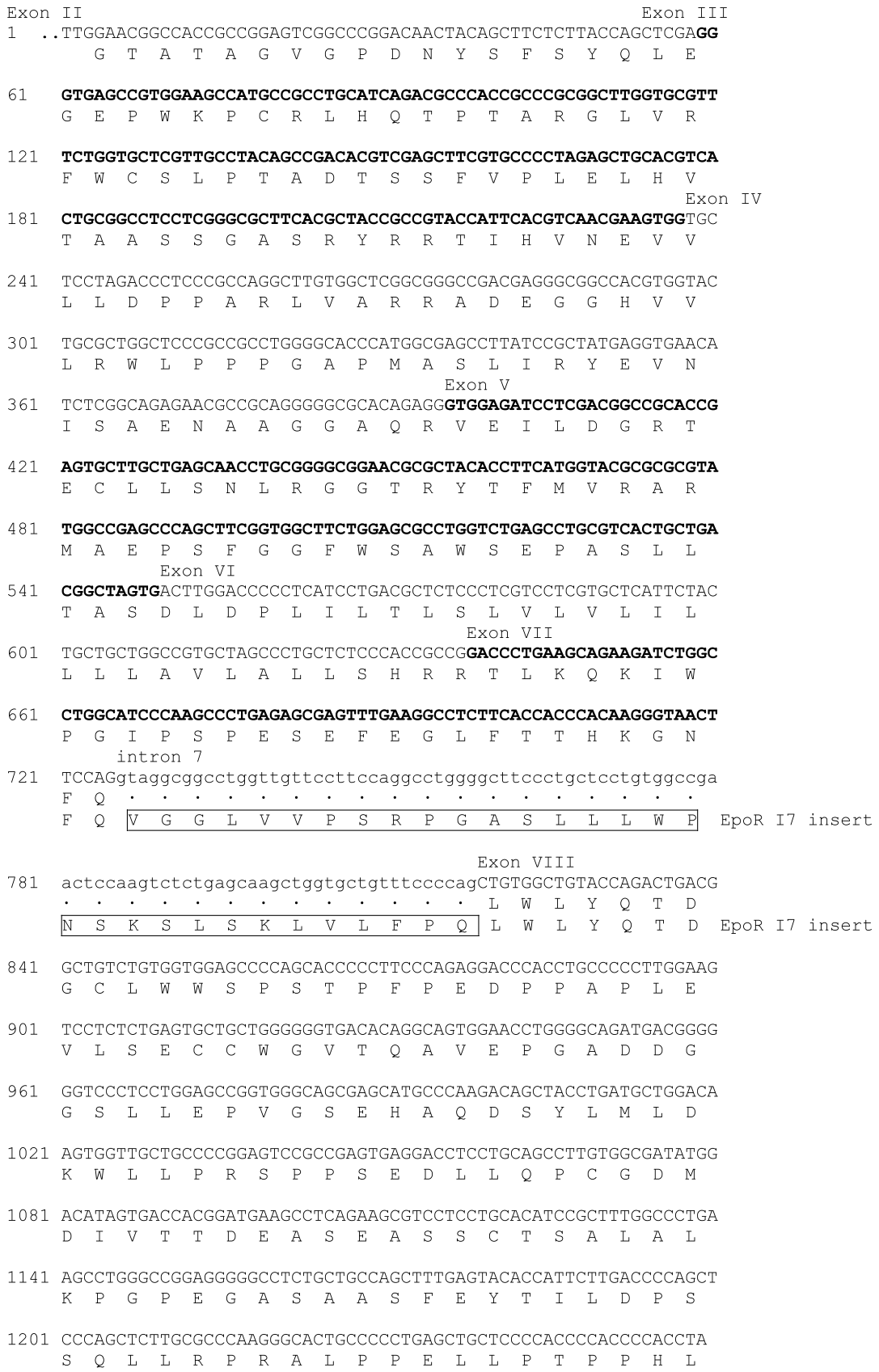

Fig. 2. Nucleotide sequence of the sheep EpoR partial cDNA. Alternating exons are in bold letters. The sequence of the 93 nucleotide insert between exon VII and VIII, found in the larger molecular weight band, is in lower-case letters. The deduced amino acid sequence is represented by the single letter codes below the nucleotide sequence. The deduced amino acid sequence of the inserted region is shown within the box.

```
1            15 16           30 31           45 46           60 61           75 76           90
SHEEP                                                                      GTATAGVGPD NYSFSYQLEGEPWKP
COW                                                                     DL VCFWEEA-------- ---------------
PIG    MYHFGATLWPGVGSL CLLLAGATWAPSPNS PDAKFESKAALLAAR GPEELLCFTERLEDL VCFWEEAGS-----E D--------------
MAN    MDHLGASLWPQVGSL CLLLAGAAWAPPPNL PDPKFESKAALLAAR GPEELLCFTERLEDL VCFWEEA-S-----G ---------D----L
MOUSE  MDKLRVPLWPRVGPL CLLLAGAAWAPSPSL PDPKFESKAALLASR GSEELLCFTQRLEDL VCFWEEA-SS-MD.F -----------SR-S
RAT    MDQLRVARWPRVSPL CLLLAGAAWASSPSL PDPKFESKAALLASR GSEELLCFTQRLEDL VCFWEEA-NS-M-.F -----------SR-S

       91          105 106         120 121         135 136         150 151         165 166         180
SHEEP  CRLHQTPTARGLVRF WCSLPTADTSSFVPL ELHVTAASSGASRYR RTIHVNEVVLLDPPA RLVARRADEGGHVVL RWLPPPGAPMASLIR
COW    -----A--------- --------------- --------.----H- --------------- --------------- ---------------
PIG    -H---G-----S--- --------------- --R--EV----P--H -I--I---------- G-L----E-S----- ---------------
MAN    -----A-----A--- --------------- --R-----.--P--H -V--I-------A-V G----L---S----- ------ET--T-H--
MOUSE  -S---A--V--S--- --------------- --Q--E-S.-SP--H -I--I-------A-- G-L----E--S---- ----------TTH--
RAT    -----A--V--SM-- --------------- --Q--E-S.-SP--H -I--I-------A-- G-L----E--S---- ----------TTH--

                                                                                    Transmembrane
       181         195 196         210 211         225 226         240 241         255 256         270
SHEEP  YEVNISAENAAGGAQ RVEILDGRTECLLSN LRGGTRYTFMVRARM AEPSFGGFWSAWSEP ASLLTASDLDPLILT LSLVLVLILLLLAVL
COW    ------------S-- --------------- --------------- --------------- --------------- ---------------
PIG    ------T------V- -----------V--- --------------- --------------- --------------- ---I-----------
MAN    ---DV--G-G--SV- -----E-----V--- ---R-----A----- --------------- V----P--------- ---I--V--V--T--
MOUSE  ---DV--G-R---T- ---V-E-----V--- ---------A----- -----S--------- --------------- ---I----S---T--
RAT    ---DV--G-R---T- ---V-E-----V--- ---------A----- -----S--------- --------------- ---I----S---T--

       271         285 286         300 301         315 316         330 331         345 346         360
SHEEP  ALLSHRRTLKQKIWP GIPSPESEFEGLFTT HKGNFQLWLYQTDGC LWWSPSTPFPEDPPA PLEVLSECCWGVTQA VEPGADDGGSLLEPV
COW    --------------- ---
PIG    --------------- ------G-------- --------------- -----C---A----- -------R------- ---A---E-------
MAN    -------A------- --------------- -----------N--- -----C---T----- S------R---TM-- ----T--E-P-----
MOUSE  ---------Q----- --------------- ---------L-R--- -----GSS------- H------PR-A---- GD-----E-P-----
RAT    -------A-R----- ------N-------- ---------L-R--- ------S-------- H------RR------ GDA--E-K-P-----

       361         375 376         390 391         405 406         420 421         435 436         450
SHEEP  GSEHAQDSYLMLDKW LLPRSPPSEDLLQPC GDMDIVTTDEASEAS SCTSALALKPGPEGA SAASFEYTILDPSSQ LLRPRALPPELLPTP
COW
PIG    -----R-T--V---- ----R-A----P--G --L-MAAM------- F-S------------ --------------- --------A--P---
MAN    -------T--V---- ----N------PG-G -SV---AM--G---- --S----S--S---- --------------- ----WT-C---P---
MOUSE  -------T--V---- ----T-C--N-SG-G -SV-P--M-----T- --P-D--S--R---T -PS------------ --C---------P---
RAT    ---R---T--V--E- ----C-C--N-SG-G DSV-PA-M--G--T- --P-D--S--R---T -PS-----------K --C---------P---

       451         465 466         480 481         495 496         510
SHEEP  PHL
COW
PIG    ---KYLYLVVSDSGI STDYSSGGSQETQGG SSSGPYSNPYENSLV PAPEPSPPNYVTCS    509
MAN    ---KYLYLVVSDSGI STDYSSGDSQGAQGG LSDGPYSNPYENSLI PAAEPLPPSYVACS    508
MOUSE  ---KYLYLVVSDSGI STDYSSGGSQGVHGD SSDGPYSHPYENSLV PDSEPLHPGYVACS    507
RAT    ---KYLYLVVSDSGI STDYSSGGSQGVHGD SSDGPYSHPYENSLV PDTEPLRPSYVACS    507
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Fig. 3. Alignment of the deduced partial amino acid sequence for sheep EpoR (388 residues) with previously reported EpoR sequences. The reported sequence for the cow is also partial. A dash denotes identity with the sheep amino acid residue, while a dot represents a gap. Amino acid residues are presented when mismatches occur compared with the different species.

Exon VII and VIII. Sequencing results showed that the shorter DNA fragment of 560 bp was the ovine EpoR cDNA. The longer DNA fragment shared the same sequence as the shorter fragment, as well as an additional 93 bp inserted between Exon VII and VIII. The partial ovine EpoR cDNA fragment containing this additional sequence has been assigned with the accession number AY029231. In-frame translation of the sequence indicated that the protein product would be an elongated protein, with additional 31 amino acid residues in the intracellular domain. No other additional bands were detected between Exon II and VIII.

The ovine EpoR sequence was highly conserved between species. At the nucleotide level, there was 97% homology with the bovine EpoR over the 686 bp of partial cow cDNA sequence available (Genbank Accession No. V61399), 85% homology with the corresponding region of human EpoR coding sequence (972 of

1141 bp, M60459), 79% homology with mouse EpoR mRNA (JO4843) and 78% with rat EpoR mRNA (D13566). At the amino acid level, there was very high homology between species—98% identity with the cow EpoR, 92% similarity with the pig EpoR, 86% similarity with the EpoR for man and mouse, and 84% for the rat (Fig. 3). In addition, significant similarity was observed for the extracellular domain of the sheep EpoR with other cytokine receptors from various species, particularly the prolactin receptor and the interleukin receptors.

3.3. Comparison of levels of expression of Epo and EpoR mRNA levels in liver kidney and placenta

No Epo or EpoR mRNA was detected in the placenta at 27 or 45 days. As shown in Table 1, a relatively constant low level of Epo mRNA (ΔC_T value ~ 24) was seen in the placenta at the three stages of gestation. The values for ΔC_T in the kidney and liver were lower, indicating higher levels of mRNA expression. There was a significant decrease ($P < 0.05$) in the Epo mRNA levels in the kidney at 140 days, and in the liver at 99 days. The kidney expressed 50–500 times as much Epo mRNA as the placenta, whilst the liver contained 9–50 times as much.

3.4. EpoR expression

EpoR mRNA was expressed at constant levels in the placenta, as seen in Table 1, at levels lower even than those of the kidney. EpoR mRNA levels were high in the liver at 66 and 99 days, which coincide with the times at which the liver is the only site of erythropoiesis, but fell to low levels near term ($P < 0.05$). EpoR mRNA was present at constant levels throughout gestation in the kidney. EpoR gene expression in the placenta was less than 0.5% of that in the liver for most of gestation.

3.5. Epo and EpoR in the hippocampus

The values of Epo and EpoR mRNA in samples of hippocampus from four ovine fetuses, at 130 days of gestation were compared with those in the liver, kidney and placenta of term fetuses (140 days of gestation). The hippocampus did express the Epo gene at low levels, at 0.07 of that in term kidney, but at six times (6 ± 3) that of the placenta. The concentration of EpoR mRNA in the hippocampus was equivalent to that of term liver/kidney, and eight times (8 ± 3) that of placenta.

3.6. Site of EpoR gene expression in the kidney

As shown in Fig. 4, EpoR mRNA was expressed in the proximal tubules of both the mesopheric and metanephric kidney at 42 days of gestation.

4. Discussion

4.1. Sheep EpoR

In this study, we report a partial cDNA sequence for the sheep EpoR. The ovine EpoR is highly homologous to known EpoR sequences of other species, particularly homologous to the bovine and porcine EpoR sequences. Different receptor isoforms have been reported for the EpoR from man, mouse and rat, arising from alternate splicing events (Nakamura et al., 1992; Kuramochi et al., 1990; Yamaji et al., 1996). A soluble receptor is expressed in man and mouse, due to the retention of part of intron four in man and intron five in the mouse. A truncated receptor is also expressed in man and rat, arising from the retention of intron seven and intron five, respectively. These truncated membrane-bound receptors may modulate target cell responsiveness to Epo, as they can bind the ligand but are

Table 1
Epo and EpoR mRNA levels in the developing ovine kidney (K) and liver (L) relative to the placenta (P)[a]

Age (days)	ΔC_T (K)	ΔC_T (L)	ΔC_T (P)	Ratio (K/P)	Ratio (L/P)
Epo					
66	16 ± 0.7	18.9 ± 0.6	24.3 ± 0.3	215 (116–867)	50 (11–90)
99	15.4 ± 0.4	20.5 ± 1.0	24.2 ± 1.4	528 (113–765)	17 (3–39)
140	17.6 ± 0.5	19.7 ± 0.4	23.5 ± 0.7	46 (15–204)	9 (5–14)
EpoR					
66	15.6 ± 0.3	9.4 ± 0.4	17.1 ± 0.5	3 (2–5)	288 (120–343)
99	15.1 ± 0.7	9.3 ± 1.0	17.1 ± 0.6	5 (2–7)	278 (63–541)
140	15.1 ± 0.9	15.1 ± 0.4	17.5 ± 0.4	7 (2–10)	7 (3–15)

[a], ΔC_T values were calculated from the formula C_T (Epo or EpoR) $- C_T$ (18S). $\Delta\Delta C_T$ was achieved as follows: $\Delta\Delta C_T = \Delta C_T$(kidney/liver) $- \Delta C_T$ (placenta). The ratio was achieved using the equation; $2^{-\Delta\Delta C_T}$. ΔC_T values are the mean $\pm$ S.E.M. Ratio values are the median (range).

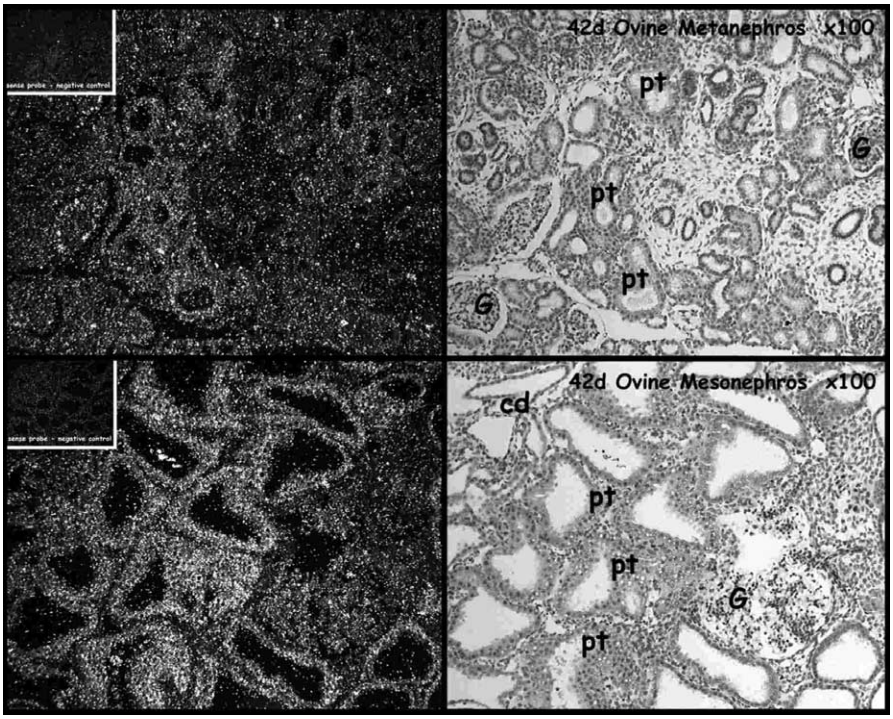

Fig. 4. Hybridization histochemistry for EpoR in kidneys (mesonephros, lower panel; metanephros, upper panel) from an ovine fetus at 42 days of gestation. Left hand side shows dark field (inset, negative control with sense riboprobe) display white dots show area of mRNA expression in proximal tubules (pt); adjacent to glomerulus (G); collecting duct (cd). Magnification $\times 100$.

not able to transduce the signal (Nakamura and Nakauchi, 1994; Yamaji et al., 1998).

The sheep EpoR also has a splice variant, with the retention of 93 bp between exons VII and VIII. The human intron seven splice variant has a 95 bp insert, which encodes a stop codon within the intronic region, giving rise to a truncated receptor (Nakamura et al., 1992). However, the same isoform in the sheep would be expected to code for a longer receptor molecule, as there are no stop codons and the translation would still be in-frame. As the previously reported splice variants resulted in truncated receptors, it is not known what role an elongated receptor would play, if any. Further studies would have to be performed to ascertain whether this elongated EpoR is produced, and if so, how Epo signal transduction is modified by this EpoR isoform. Preliminary studies suggest that the expression of the longer transcript is slightly higher in the bone marrow of the neonatal lamb (1–10 weeks old), than in the liver of the fetus at 60–100 days (Lim, unpublished results).

4.2. Techniques used

Whilst it has long been known that the kidney is the major source of systemic Epo in the adult mammal (Jacobson et al., 1957), it was not until the cellular location of mRNA was possible by the technique of in situ hybridization histochemistry that the precise renal cell type synthesizing Epo was elucidated (Koury et al., 1988; Lacombe et al., 1989). Prior to that time, studies using antibodies to Epo had (incorrectly) pointed to glomeruli and to tubular cells as the cells of origin. With the technique of in situ hybridization histochemistry, the true site of Epo production in the adult kidney has been shown to be some of the interstitial cells in the vicinity of the proximal tubules in the mouse (Koury et al., 1989; Lacombe et al., 1988; Maxwell et al., 1993), rat (Eckardt et al., 1993), sheep (Darby et al., 1995), monkey (Fisher et al., 1996) and human (Liapis et al., 1995). This is important as it is now well recognized that Epo is not stored, even in the kidney, which is why it is necessary to study the regulation of production at the level of gene transcription.

The first real quantitation of Epo mRNA levels was carried out with the technique of competitive PCR in the sheep (Fu et al., 1993; Lim et al., 1994), rat (Fandrey and Bunn, 1993) and human (Dame et al., 1998, 2000). The technique of real-time PCR is even more sensitive, requiring 50 ng or less of cDNA, and exhibits good reproducibility. The coefficient of variation of 11% for Epo compares favorably with the CV of 14%

for fetal renal renin determination, though not as good as the 5–7% for the angiotensin receptors, and aquaporins 1 and 3 (Moritz et al., 2000; Johnston et al., 2000). Even with this sensitive technique, the levels of Epo mRNA in the placenta were not detectable early in gestation in sheep, and when compared with those of fetal liver and kidney, were very low throughout gestation. Recently, it has been suggested that the binucleate cell of the fetal trophoblast, which migrates into the maternal side of the ovine placenta, is the site of Epo production (Kim et al., 2001).

4.3. Ontogeny of Epo gene expression

As reported previously, Epo gene expression was much higher in the kidney than the liver at all times, and Epo gene expression decreased in the liver earlier than in the kidney (Lim et al., 1994). Epo gene expression has been shown to be higher in the fetal pig liver at 24 days of gestation (term $\sim$112 days) than at 30 days, and barely detectable at 40 days (Klemcke et al., 2001). This differs from the situation in the human fetus, where Epo gene expression, whilst present in the fetal kidney for much of gestation, is never as high as in the fetal liver (Dame et al., 1998). In the human placenta, Epo mRNA could not be detected by Northern blotting with 50 μg of total RNA, but a PCR product was produced from 20 μg of cytotrophoblast mRNA from term placenta (Conrad et al., 1996). At the low level found in the ovine placenta, it was not possible to locate the cell of origin. Even with the sensitive technique used (real-time PCR) it was not possible to detect expression at 27 and 45 days, whereas Epo gene expression does occur in the mesonephros at these early stages of gestation (Wintour et al., 1996). It is of interest to note that placental factors, other than Epo, have been described which can influence erythropoiesis (Petraglia et al., 1987; Shao et al., 1992; Socolovsky et al., 1998; Bittorf et al., 2000) and these could belong to the prolactin, activin, inhibin or trophoblast-specific cytokine families. Some of these are also regulated by hypoxia (Jenkin et al., 2001). However, none of these factors can compensate sufficiently to prevent the lethal effects when the Epo gene itself is not expressed.

4.4. Ontogeny of EpoR

EpoR is expected to be found in the fetal liver whilst it is the site of haemopoiesis, which in the fetal sheep is up until 130 days of gestation (Moritz et al., 1997) and in the bone marrow thereafter. The low level of EpoR expression in the liver, at 140 days, may reflect hepatocyte/vascular sites of expression. EpoR mRNA is also detected in early gestation in porcine (Pearson et al., 2000) and human fetal liver (Juul et al., 1998). The presence of EpoR mRNA in the fetal kidney, at relatively low but invariant levels from 66 to 140 days of gestation, is consistent with the finding of EpoR mRNA at low levels in the human, rat and mouse kidney (Westenfelder et al., 1999). The function of EpoR in these proximal tubular cells is unknown, but EpoR is expressed in renal carcinoma cells and activation stimulates the proliferation of these cells (Westenfelder and Baranowski, 2000).

4.5. Epo/EpoR in reproductive tissues

EpoR in the placenta has been localized to the endothelial cells of the blood vessels alone (Anagnostou et al., 1994) or to the trophoblast cells and blood vessels (Fairchild Benyo and Conrad, 1999). In this study, no distinct localization was possible in the placenta of sheep. With antibodies to the EpoR it has been claimed to be quite widespread in the interdigitating fetal-maternal tissue (Kim et al., 2001). Very recently, Epo mRNA was detected, by Northern blot, in a few placental samples from early gestation piglets (Klemcke et al., 2001).

It is worthy of note that the uterine endometrium expresses EpoR (Masuda et al., 1999) and the uterus expresses Epo (Yasuda et al., 1998). This would suggest that Epo has a paracrine effect in the uterus. The angiogenic effect of Epo (Anagnostou et al., 1990, 1994) may possibly play a role in implantation, the attachment of the trophoblast to the uterine lining, by stimulating angiogenesis in both the endometrium and the trophoblast cells of the embryo.

4.6. Epo/EpoR in the brain

The other major organ in which both Epo and EpoR have been found is the brain (Liu et al., 1997; Marti et al., 1996). Epo is produced by astrocytes (Masuda et al., 1994) and the mature protein is said to be smaller than that produced in the kidney, due to less sialylation of the molecule. In one study, it was claimed that the mRNA for Epo, but not for EpoR, was up-regulated by hypoxia (Digicaylioglu et al., 1995). When a human EpoR transgene was expressed in the mouse, it appeared in the brain, as well as the embryonic yolk sac, liver adult bone marrow and spleen (Liu et al., 1997). In the current preliminary study, Epo mRNA was detected in the hippocampus of late gestation ovine fetuses, at levels much lower than that of the kidney, although at levels much greater than that in the placenta.

The EpoR made by brain capillary endothelial cells (rat) exists in two forms, the majority being a form that includes intron five, thereby encoding for a trun-

cated receptor protein, as well as a minority ($\approx 20\%$) of the authentic EpoR (Yamaji et al., 1998). EpoR is also found on neurons, particularly in the hippocampus and cerebral cortex (Digicaylioglu et al., 1995), Morishita et al., 1997). Exposure of hippocampal neurons from late gestation rats, cultured for 7–10 days, to low doses of Epo (3–30 pM for as little as 5 min) at least 8 h prior to insult, protects them from glutamate (1 mM) induced neuronal death (Morishita et al., 1997). In hippocampal cell cultures from 5-day-old rats, Epo (100 pM) also caused a significant decrease in hypoxia-induced cell death (Lewczuk et al., 2000). During in vivo experiments in gerbils, the intracerebroventricular infusion of Epo prevents the delayed neuronal death in the hippocampal CA1 field following ischaemia (Sakanaka et al., 1998). This same group later showed, in rats, that Epo could prevent place navigation disability when the middle cerebral artery was occluded (Sadamoto et al., 1998). In the ovine fetal hippocampus the level of EpoR was roughly equivalent to that in the late gestation kidney and liver, but greater than that of the placenta.

5. Summary

This paper describes the isolation of the first partial clone of the ovine EpoR and the fact that it is expressed in both the fetal kidney and the placenta, albeit at levels much lower than in the erythroid tissues (liver, 66–99 days). Epo mRNA is found in the ovine placenta, from at least 66 days onwards. However, the expression levels are extremely low compared with that of the hippocampus, kidney and liver.

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