EXHIBIT O

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Erythropoietin production by interstitial cells of hypoxic monkey kidneys

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Summary. Previous studies which demonstrated that interstitial cells of the peritubular capillary bed of the kidneys are the site of erythropoietin (Epo) production have been performed in non-primate species. In this study, kidneys from adult rhesus monkeys exposed to 18 h hypoxia (0·42 atm) with high serum (5685 mU/ml) and kidney (814 mU/g, includes serum EPO in the kidney) levels of Epo were compared with a kidney from a nonhypoxic normal rhesus monkey. Localization of Epo mRNA by *in situ* hybridization was carried out with either anti-sense or sense RNA probes generated from a 645 base pair KpnI±BglII fragment of a monkey Epo cDNA. Epo mRNA was demonstrated only in interstitial cells in the peritubular capillary bed of the hypoxic and normal monkey kidneys utilizing the antisense probe. The finding that the same type of cell that produces EPO in mice, rats and sheep also produces EPO in a higher primate species strongly supports the contention that renal interstitial cells also produce EPO in the human.

Keywords: erythropoetin, kidney, monkey, hypoxia, interstitial cells.

Erythropoietin (Epo) is a glycoprotein hormone produced by the kidney and liver in the adult human which regulates red cell production (Fisher, 1993). Since the human, monkey, mouse and sheep Epo gene have all been cloned (Jacobs *et al*, 1985; Lacombe *et al*, 1988b; Lin *et al*, 1985; McDonald *et al*, 1986; Shoemaker & Mitsock, 1986; Fu *et al*, 1993), cDNA probes for these species have been developed to localize Epo mRNA. Most previous studies involving the localization of Epo mRNA in the kidneys of mice and rats using *in situ* hybridization and/or transgenic technology have suggested that peritubular interstitial cells as the source of Epo (Bachmann *et al*, 1993; Koury *et al*, 1988, 1989; Lacombe *et al*, 1988a; Schuster *et al*, 1992; Semenza *et al*, 1991a; Maxwell *et al*, 1993); however, two studies have reported that tubular epithelial cells are the source of Epo (Loya *et al*, 1994; Maxwell *et al*, 1990). One of these studies, identifying tubular cells as the source of Epo (Maxwell *et al*, 1990), was performed under conditions in which the resolution of the *in situ* autoradiograms was considered to be questionable (Koury *et al*, 1991a). Recently, peritubular interstitial cells were found to produce Epo in fetal and adult sheep kidneys as well (Darby *et al*, 1995). Localization of Epo mRNA in discrete cells of a normal primate kidney has not previously been reported. But cells in the cyst wall of polycystic human kidneys have been reported to produce Epo (Eckardt *et al*, 1989). In the present study, Epo production in hypoxic and nonhypoxic monkey kidneys was evaluated using *in situ* hybridization with a monkey-specific Epo cDNA probe.

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MATERIALS AND METHODS

Hypoxic stimulation, fixation and processing of tissues from rhesus monkeys. Two rhesus monkeys were obtained from the Tulane Regional Primate Center in Covington, Louisiana. The haematologically normal non-hypoxic male rhesus monkey was anaesthetized with pentobarbital anaesthesia and the right kidney removed through a retroperitoneal incision. A portion of the kidney was immediately frozen in liquid nitrogen and stored at −70 C until sectioned for microscopic studies. Another portion of this kidney was placed in 10% formalin and preserved for later embedding in paraffin. A sample of blood was taken post-surgically to determine the haematocrit and serum Epo levels. The haematocrit of this normal monkey was 38%, which is in the normal range for rhesus monkeys at the Tulane Primate Center (34·3 ± 4·13%). A second (female) rhesus monkey was placed in a hypobaric chamber (0·42 atm) for 18 h and the following organ specimens were taken under pentobarbital anaesthesia immediately after the monkey was removed

from the hypobaric chamber: kidney, liver, lung, brain, skeletal muscle, and spleen. A sample of blood was taken before exposure to hypoxia and immediately upon removal from the hypobaric chamber, for determination of the haematocrit and serum Epo levels. After determining the haematocrit, the blood sample was allowed to clot, centrifuged at 1000 rpm and the serum removed for Epo analysis using a sensitive radioimmunoassay (RIA) for Epo (Garcia *et al*, 1990). The linear range of this RIA was between 5 and 400 mU/ml. The mean and standard error of the mean for the assay of Æve samples from the same organ are shown in Table I. Table I also shows the results of the haematocrit, body weight and serum and kidney levels of Epo in the 18 h hypoxic and control monkeys. The haematocrit of the female monkey was 49% before, and 50% following, exposure to hypoxia and sacriÆce, which is above the normal range for rhesus monkeys at the Tulane Primate Center. It is possible that this monkey was slightly dehydrated. Epo was extracted from the kidney as described previously (Katsuoka *et al*, 1983). The amount of Epo contained in the kidney was estimated to be 814 mU/g and after correcting for the estimated amount of Epo in the plasma in the kidney (238 mU/g), Epo levels were 576 mU/g (Snyder *et al*, 1974). No detectable Epo was found in skeletal muscle.

In situ hybridization. Tissue sections were cut from paraffin blocks at a thickness of 3± 5 m, deparaffinized and treated with proteinase K and acetic anhydride as previously described (Koury *et al*, 1988). Slides contained either one section of kidney taken from the hypoxic monkey and one section of kidney taken from the normoxic monkey, or liver, spleen and skeletal muscle from the hypoxic monkey. A 645 base pair *KpnI-BglII* arrangement of monkey Epo cDNA was subcloned into the plasmid vector pGEM 4Z (Promega Biotec, Madison, Wis.) and used to generate both sense and antisense RNA probes. [-^{33}P]uridine 5 -triphosphate (3000 Ci/mmol) (Dupont/NEN, Boston, Mass.) was used to label the probes. An identical fragment of human Epo cDNA has previously been used in studies to localize human Epo mRNA in transgenic mice (Koury *et al*, 1991a). Duplicate sections from the same area of both the hypoxic and non-hypoxic kidney were hybridized with either the antisense probe to detect Epo mRNA, or the sense probe to detect non-speciÆc hybridization. Hybridization, post-hybridization washes, autoradiography and documentation of morphology of Epo-producing cells by the periodic acid± Schiff reaction were performed as previously described (Koury *et al*, 1991a).

The animal protocol was approved by The Tulane University Institutional Advisory Committee for Animal Resources.

RESULTS

In situ hybridization

Kidney tissue taken from the monkey exposed to 18 h of hypoxia were subjected to *in situ* hybridization using the antisense Epo probe in order to localize cells producing Epo mRNA. Epo mRNA was localized in interstitial cells throughout the kidney cortex (Figs 1A and 1B). No Epo mRNA was localized in either glomerular or tubular cells (Fig 2). No such cells were detected when the sense strand probe was used on either hypoxic or normoxic kidneys (not shown). Glomeruli exhibited only background hybridization with the probe (Fig 1). Figs 2A and 2B both document the interstitial cell localization of the Epo-producing cells by the demonstration of Epo mRNA in these cells. Cells that were overlaid by silver grains in Fig 1A are clearly shown to lie outside of the tubular cell basement membranes and glomerular tuft as seen in Fig 1B and Figs 2A± D.

Both the antisense and sense strand probes were used on liver, spleen and skeletal muscle of hypoxic monkeys in order to determine if Epo-producing cells could be localized in any of these tissues. No Epo-producing cells were detected in any of these tissues, even after 6 weeks of exposure to autoradiograms (not shown). An occasional interstitial cell containing Epo mRNA was seen in the kidney from the normal non-hypoxic monkey.

Tissue levels of erythropoietin

As noted in Table I, the kidney levels of Epo in the 18 h hypoxic monkey were 814 mU/g, and even when corrected for the estimated amount of plasma (using the haematocrit and estimated blood volume of each monkey) contained in the kidney, the kidney Epo levels were 576 mU/g. The Epo levels in the normal monkey kidney were undetectable. Epo

Table I. Serum erythropoietin, body weight and haematocrit values in normal and hypoxic* monkeys.

	Body wt (kg)	Sex	Haematocrit (%)	Serum levels of Epo (mU/ml)		Kidney Epo levels (mU/g)
				Pre-hypoxic	Post hypoxic	
Normal	7·6		38	4·4	Ð	N.D.
Hypoxic (18 h)	9·6		49	5·7	5786·6	814 271

*The hypoxic rhesus monkey was exposed to hypoxia (0·42 atm) for 18 h in a hypobaric chamber.

Haematocrit was taken post-surgically immediately after the kidney was removed under pentobarbital anaesthesia. The kidney Epo levels (814 271 mU/g) includes the plasma contained in the kidney. Even when the kidney levels of Epo were corrected for approx. 41 l plasma/g (Snyder *et al*, 1974) contained in the kidney (238 mU/g), it is estimated to contain approx. 576 mU Epo/g. Epo levels in skeletal muscle were undetectable.

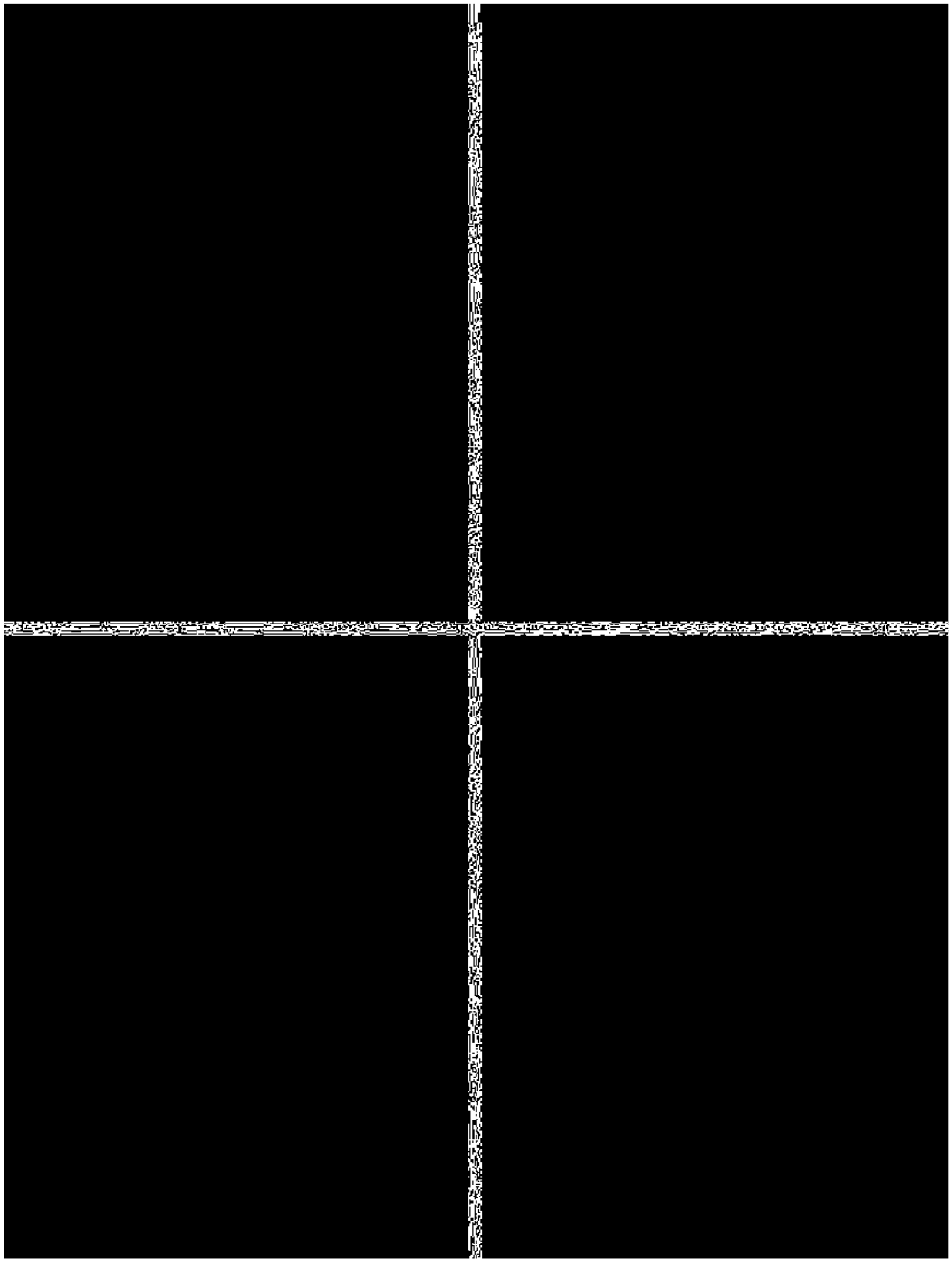
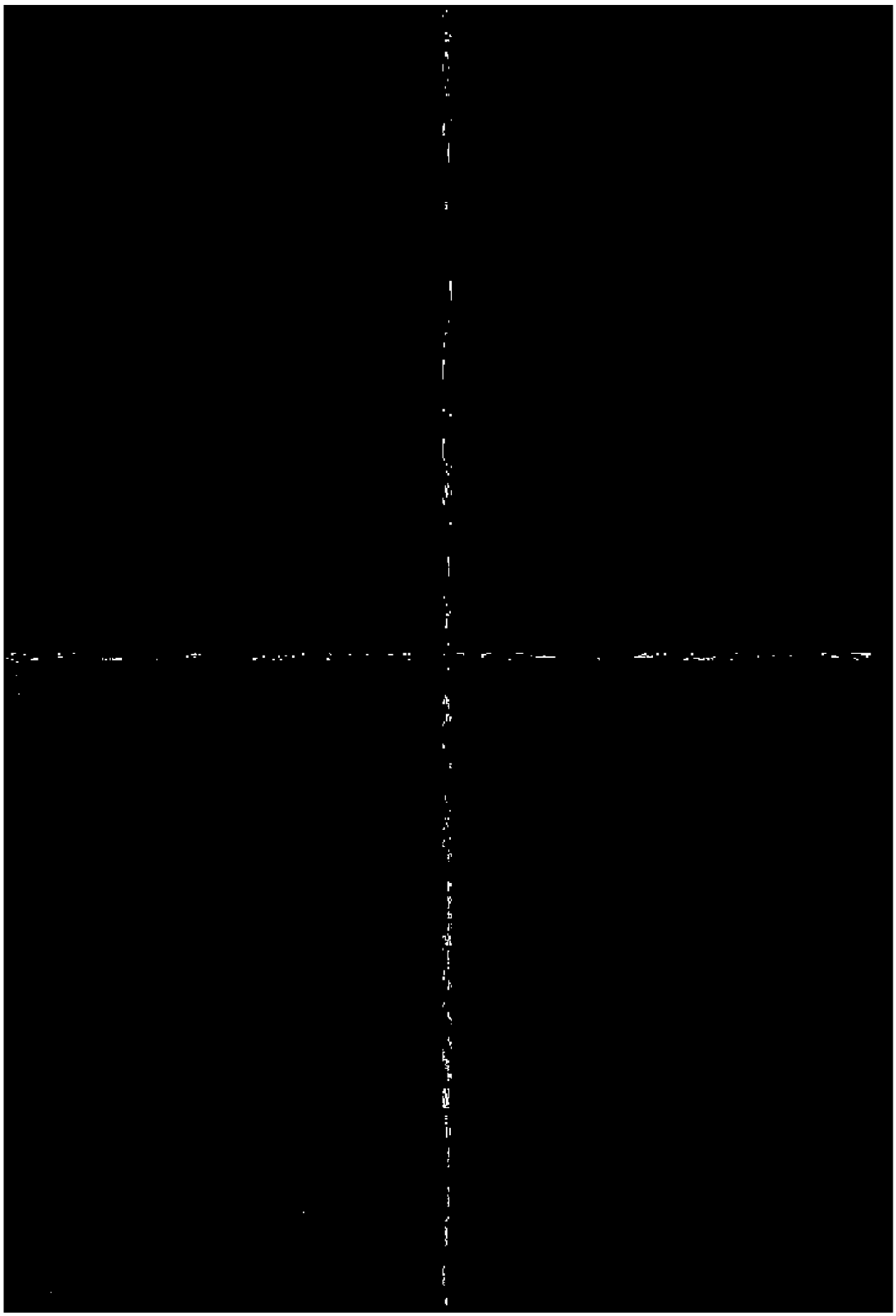


Fig 1. Localization of Epo-producing cells. Dark-field (A and C) and corresponding bright-field (B and D) images of hypoxic (A and B) and nonhypoxic (C and D) kidney sections hybridized with the antisense Epo probe. Bar 50 m. G, glomerulus. Three obvious peritubular aggregates of silver grains are indicated in A and B (a, b and c).



levels in the skeletal muscle extract of the hypoxic monkey were undetectable.

DISCUSSION

The data in the present report demonstrates that messenger RNA for Epo in hypoxic monkey kidneys is expressed only in the cells of the peritubular interstitium. Peritubular interstitial cells have been previously identified as Epo-producing cells in kidneys of normal (Bachmann *et al*, 1993; Koury *et al*, 1988, 1989; Lacombe *et al*, 1988a) and transgenic (Maxwell *et al*, 1993; Semenze *et al*, 1991a) mice, in rats (Schuster *et al*, 1992) and in sheep (Darby *et al*, 1995). It has been proposed that these interstitial cells are fibroblasts, based on immunohistochemistry at the light and electron microscopic levels. Bachmann *et al* (1993) used a combination of high-resolution interference contrast optics and co-localization of Epo mRNA and ecto-5-nucleotidase to demonstrate that peritubular fibroblasts produce Epo in normal mice. Maxwell *et al* (1993) localized expression of a Epo-SV 40 T antigen transgene to a similar cell population, using both light and electron microscopy (Bachmann, 1993; Maxwell *et al*, 1993). A recent study by Loya *et al* (1994) has reported that transgenic mice carrying the Epo gene promotor linked to *lacZ* express the reporter in proximal convoluted tubule cells, rather than in peritubular interstitial cells after hypoxia. The reasons for the discrepancy between the study of Loya *et al* (1994) and those of Semenza *et al* (1991a) and Maxwell *et al* (1993) could be due to the fact that the transgene used by Loya *et al* (1994) contained only approximately 6 kb of 5 flanking sequence. In contrast, Semenza *et al* (1991a) found interstitial cell expression in transgenic animals having 13·5 and 1 6·5 kb of 5-flanking sequence. On the other hand, Maxwell *et al* (1993) used a construct containing approximately 9 kb of 5 flanking sequences. Thus, it is possible that an important element that controls the cell type specific expression of Epo in interstitial cells, lying between 6 kb and 9 kb 5 to the Epo gene, was not present in the construct of Loya *et al* (1994). Furthermore, constructs used by Semenza *et al* (1991a) contained between 0·3 and 2 ·2 kb of 3 flanking sequence, whereas the construct used by Maxwell *et al* (1993) contained more than 3 kb of 3 flanking sequence. All of these constructs contained a known 3 hypoxia inducible enhancer element (Semenza *et al*, 1991b; Beck *et al*, 1991; Pugh *et al*, 1991). The Epo/LacZ construct used by Loya *et al* (1994) contained no 3 sequence, and thus lacked the known hypoxia inducible enhancer element.

The specificity of our *in situ* hybridization results in the kidneys is supported by the finding that no specific hybridization was detected when the sense strand probe was used on either the hypoxic or normal kidney. Our finding of an occasional interstitial cell with Epo mRNA in normal monkey kidneys is consistent with the studies reported in mice (Koury *et al*, 1989) and rats (Schuster *et al*, 1992), where small numbers of Epo-producing cells were detected in the kidneys of nonhypoxic or nonanaemic animals.

No Epo mRNA was detected in the liver, spleen or skeletal muscle of the hypoxic monkey. The inability to detect Epo-producing cells in the liver was somewhat surprising, because a mixed population of hepatocytes and nonparenchymal cells have been observed to contain Epo mRNA in both mice (Koury *et al*, 1991b) and rats (Schuster *et al*, 1992) using *in situ* hybridization. Further, primary cultures of purified rat hepatocytes have been shown to produce Epo mRNA (Schuster *et al*, 1992; Eckhardt *et al*, 1993) and an Epo-SV40 antigen fusion gene product has been localized in both nonparenchymal ITO cells and a subset of hepatocytes in transgenic mice (Maxwell *et al*, 1994). The inability to detect Epo-producing cells in the livers of hypoxic monkeys could have been due to a number of factors. These could include insufficient exposure time of the *in situ* autoradiograms, a very low level of expression of Epo mRNA by the majority of hepatocytes, or regional differences within the liver with regard to Epo production. Since the liver of a monkey is much larger than that of either a mouse or rat, multiple sections of tissue from different areas of the hypoxic liver would need to be analysed in future *in situ* hybridization experiments in order to rule out this possibility. It is also possible that very little Epo is produced in the primate liver as compared to rodents.

As stated above, the majority of studies involving the localization of Epo mRNA by *in situ* hybridization have used rodent models. One of these studies involved the use of transgenic mice carrying a human Epo transgene. Semenza *et al* (1991a) found that human Epo mRNA was specifically expressed in peritubular interstitial cells in two of the transgenic mouse lines studied. Although those results suggested that Epo might be produced in peritubular cells in humans as well as mice, no *in situ* hybridization studies involving localization of Epo in normal human kidneys have been published. Epo mRNA has been reported in the wall of kidney cysts of polycystic human kidneys (Eckardt *et al*, 1989) and this finding is consistent with the hypothesis that Epo-producing cells in human kidneys are probably derived from an interstitial, fibroblast-like cell (Bachmann *et al*, 1993). However, the kidney architecture was too disrupted in the polycystic kidney to determine if the Epo-producing cell was interstitial cells. The present finding that interstitial cells produce Epo in hypoxic monkey kidneys suggests that interstitial cells in the kidneys of other primates such as the human are likely to be the primary site of Epo production as well.

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Fig 2. *In situ* hybridization localization of Epo mRNA in a hypoxic monkey kidney. (A) *In situ* autoradiogram of positive (Epo mRNA) interstitial cells in hypoxic monkey kidney. (B) Negative glomerulus (G) and positive interstitial cell. Magnification of A and B, 1114. (C) Higher magnification *in situ* autoradiogram of the hypoxic kidney. (D) Same area of kidney section as C after removal of silver grains from the autoradiogram and staining the section using para-aminosalicylate (PAS). Bar in D 20 m for C and D. Arrows in A and B, and arrowheads a, b, c and d indicate Epo mRNA positive interstitial cells.

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