Expression of progranulin during the first stages of liver development in rat Fischer 344

Expressão da progranulina durante os primeiros estágios de desenvolvimento hepático em ratos Fischer 344

Arthur C. de L. LUNA[1]; Cristiane C. PASSOS[2]; Amanda O. FERREIRA[2]; Andrew BATEMAN[3]; Maria A. MIGLINO[2]; Ricardo R. GUERRA[1]

[1] Programa de Pós-Graduação em Ciência Animal da Universidade Federal da Paraíba – CCA, PB, Brasil
[2] Programa de Pós-Graduação em Anatomia dos Animais Domésticos e Silvestres da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, São Paulo – SP, Brasil
[3] Department of Medicine, McGill University Health Centre, Royal Victoria Hospital, Que., Canada

Abstract

Transplants are the only effective therapy for the treatment of advanced liver diseases such as cirrhosis. Given the limited number of organ donors, regenerative medicine has sought for sources of cells and tissues for replacement therapy. Embryonic stem cells are a promising source of material for transplantation because of their exclusive property of being expanded indefinitely in culture, thus, they are a source of replacement tissue. Moreover, they are capable of differentiating into practically all cell types, and may be utilized in replacement therapy in various diseases. The liver bud has bipotent stem cells that have not yet differentiated into hepatocytes or biliary duct cells; however, they have great potential of proliferation and differentiation. Thus, the challenge is to identify methods that promote their differentiation in specific and functional strains. This study aimed to evaluate the role of the progranulin growth factor PGRN during the liver development of rats F344, since this growth factor could be utilized in protocols of differentiation of stem cells of the liver bud in functional hepatocytes. The results showed that PGRN is present during different periods of hepatogenesis in F344 rats, and that this growth factor should be involved in the process of differentiation of hepatoblasts into hepatocytes after activation by HNF4α , however, PGRN seems not to exert a cellular proliferation function during the hepatogenesis. Thus, PGRN can be used in future protocols of liver cell differentiation directed toward cellular therapy in Regenerative Medicine.

Keywords: Progranulin. Embryonic stem cells. HNF4α. Liver bud. PCNA.

Resumo

Os transplantes são a única terapia eficaz para o tratamento de doenças hepáticas avançadas, como a cirrose. Dado o número limitado de doadores de órgãos, a medicina regenerativa tem procurado fontes de células para a terapia de substituição. As células embrionárias são uma fonte promissora de material para o transplante devido à sua propriedade exclusiva de serem expandidas indefinidamente em cultura, assim, elas são uma fonte de tecido de substituição. Além disso, são capazes de se diferenciar em praticamente todos os tipos celulares, e podem ser utilizadas na terapia de substituição em várias doenças. O broto hepático tem células-tronco (CT) bipotenciais que ainda não se diferenciam em hepatócitos ou células do ducto biliar, contudo, elas têm um grande potencial de proliferação e de diferenciação. Desse modo, o desafio é identificar métodos que promovam sua diferenciação em linhagens específicas e funcionais. Este estudo teve como objetivo avaliar o papel do fator de crescimento progranulina (PGRN) durante o desenvolvimento hepático em ratos F344, uma vez que a PGRN poderia ser utilizada em protocolos de diferenciação de CT do broto hepático em hepatócitos funcionais. Os resultados mostraram que PGRN está presente durante diferentes períodos da hepatogênese em ratos F344, e que a mesma deve estar envolvida no processo de diferenciação de hepatoblastos em hepatócitos após ativação por HNF4α, no entanto, a PGRN parece não desempenhar uma função de proliferação celular durante a hepatogênese. Assim, a PGRN pode ser usada em futuros protocolos de diferenciação de células hepáticas voltadas para a terapia celular na medicina regenerativa.

Palavras-chave: Progranulina. Células-tronco embrionárias. HNF4α. Broto hepático. PCNA.

Correspondence to:
Dr. Ricardo R. Guerra
Laboratory of Histology and Histopathology
Programa de Pós-Graduação em Ciência Animal
Universidade Federal da Paraiba - CCA
Campus II, Areia - PB, Brasil
CEP 58397-000
e-mail: ricardo@cca.ufpb.br

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Introduction

Diseases involving endodermically derived organs, particularly the lungs, liver and pancreas, including cystic fibrosis, chronic hepatitis and diabetes respectively, affect thousands of people in the world. Notably, even though the liver has regenerative properties, most of the injuries occurring from these diseases are extremely destructive, and can cause progressive lesions in this organ, and in more severe cases, cirrhosis (ALFIERI; MIES, 2001). Thus, repair mechanisms become ineffective, and a transplant is the only option. However, the insufficient number of organ donors and the complexity of the surgical procedure, even today are still normally limiting factors for this choice of treatment. An alternative choice of treatment for these patients is the stimulation of the regeneration of the affected tissue *in vivo* or generation of replacement tissue *in vitro.* This research area is referred to as Regenerative Medicine (SPENCE; WELLS, 2007).

The use of primary hepatocytes derived from a reduced number of cells from an adult liver for cell culture could be used as a solution to the limited number of human donors. However, it has been observed that this cell line does not have a great capacity of *in vitro* proliferation. But this limiting factor is not verified in embryonic stem cells, which maintain their differentiation potential for several cell types and consequently makes them an important source for different cell types and cell therapy (PARK; LEE, 2005).

Pre-established liver strain cells, which have a greater differentiation potential, are another important source (KIYOTA; MATSUSHITA; UEOKA, 2007). Thus, the challenge is to identify methods that promote differentiation in adult and functional hepatocyte strains (SPENCE; WELLS, 2007). These strains can become a proposal for the future, not only for the treatment of liver diseases, but also in the development of new drugs (PAUTON; HAYNES, 2005).

Studies show that progranulin growth factor (PGRN) can be involved in differentiation of embryonic cells and that it is a growth factor with great potential to induce cell proliferation (HE; BATEMAN, 2003) because it is involved in embryonic and neonatal development (DANIEL et al., 2003), and thus, could be linked to the differentiation of hepatoblasts into hepatocytes.

PGRN is also involved in cell survival (GUERRA et al., 2007), in tumorigenesis (TANGKEANGSIRISIN; HAYASHI; SERRERO, 2004), in the prevention of the frontotemporal lobe degeneration (MACKENZIE et al., 2006; PETKAU et al., 2012; WHITWELL et al., 2012), and is present (protein and mRNA) in the liver and intestine of cirrhotic rats (GUERRA et al., 2009). According to Diaz-Cueto et al. (2000), inhibition of PGRN action retards blastocyst formation, and exogenous PGRN has the ability to accelerate the onset of cavitation and is a growth factor for trophectoderm epithelial cells. Suzuki et al. (2000) show that involvement in the production of hypothalamic PGRN leads to a damaged sexual differentiation of the masculine hypothalamus.

Li et al. (2012) showed that high PGRN is associated to increased angiogenesis of breast carcinoma. PGRN still acts as a promising endogenous neuro-protector with anti-apoptosis and anti-inflammatory properties utilized in recuperation of motor and neurologic functions after cerebral infarct (TAO et al., 2012). Studies also suggest that PGRN is involved in the advancement of astrocytoma and can be a prognostic biomarker for glioblastoma (YILMAZ et al., 2011). It is also associated to the degree of liver fibrosis in non-alcoholic patients (AL-AYADHI; MOSTAFA, 2011). The great diversity of functions allotted to PGRN in the past few years shows that it needs to be further studied.

Another protein, Hepatocyte Nuclear Factor-4α (HNF-4α) is a transcription factor belonging to the steroid hormone receptor subfamily (CHEN et al., 2010). Studies have demonstrated that HNF-4α acts in the cascade of transcription factors that lead to liver differentiation and is a hepatocyte marker, which was used in this study.

PCNA (proliferating cell nuclear antigen), which is involved in the mitotic processes, is essential to eukaryotic DNA replication and is considered a cell proliferation marker, and consequently, it is also present in hepatogenesis (TAN et al., 1986; BRAVO et al., 1987; PRELICH et al., 1987). PCNA was also studied in this research, and the PCNA and HNF4α sites of positivity were compared to those of the PGRN.

Thus, this study aims to elucidate the PGRN growth factor during hepatogenesis in different embryonic stages of rats *(Rattus norvegicus)* Fischer 344, with the future objective of using it as an agent for differentiating embryonic cells into hepatocytes.

Materials and Methods

Histological fixation, embedding and processing: The embryos used, at 12.5 days of development (E12.5); E13.5; E14.5; E15.5; and E16.5, were collected and given by the Division of Anatomy of the Department of Surgery of School of Veterinary Medicine and Animal Science, University of São Paulo (SVMAS- USP), Brazil, according to the requirements of its Bioethics Committee under the number of 1377/2008. The biological material was collected and fixated in Metacarn (60% methanol, 30% chloroform and 10% acetic acid).

After a 12-hour fixation, the samples were dehydrated in a series of increasing concentrations of ethanol (from 70 to 100%) and diaphanized in xylene, and then the whole embryo was embedded in paraplast® (Paraplast Embedding Media, Paraplast Plus, Sigma, Oxford Lab. USA) (JUNQUEIRA, 1995).

Serial slicing at a thickness of 5 µm, obtained on a semi-automatic microtome (Leica, RM2165), was performed on the entire embryo. The slices were adhered to histology slides and left in an oven at 60ºC.

Detection of PGRN, HNF4α and PCNA antigens: The primary antibody anti-PGRN (kindly provided by Dr. Andrew Bateman, produced in his laboratory – Endocrinology Laboratory/Victoria Hospital/Mc Gill/Canada) was used in 5 slices per embryo. The histologic sections were collected in silanized slides, dewaxed and dehydrated according to standard protocol. The slides were submitted to three one-minute distilled H_2O baths. Then the sections were submitted to Hydrogen Peroxide block during 10 minutes. This process was repeated three times, and then the sections were washed three times in phosphate buffered saline (PBS) for 3 minutes each. The slides were submitted to antigen unmasking procedure by using Citrate Buffer (pH6.0) for 10 minutes in a microwave oven, and after being heated, they were left at room temperature for 20 minutes. Later, they were washed with PBS and incubated in a humid chamber overnight at 4ºC, with antibodies diluted in PBS. The slides that received the anti-PGRN antibody were incubated with positive control, and those that received the PBS with negative control. Afterwards, they were washed and incubated with biotinylated secondary antibodies during 15 minutes, followed by incubation with Streptavidin peroxidase complex (Dako-LSAB) during 30 min. The positive cells were seen after being stained with DAB (Dako) during 5 min. For the detection of HNF4α and PCNA antigens, the same procedure described above was used, differing only by the primary antibody utilized. For PCNA detection, the duodenum of the corresponding animal was used as positive control in the reaction. The photomicrographs were performed by KS400 4.4 ZEISS brand software. An OLYMPUS BX60 microscope and an AxioCam (ZEISS) camera were used for the procedure.

Results

Immunohistochemistry for PGRN marking

The immunohistochemistry for the marking of the expression of the PGRN protein, as well as for the other antibodies, was performed and repeated for all the embryonic stages proposed in this study. Anti-PGRN showed positivity in all the stages verified (Figure 1), whereas age E12.5 (Figure 1B) was less

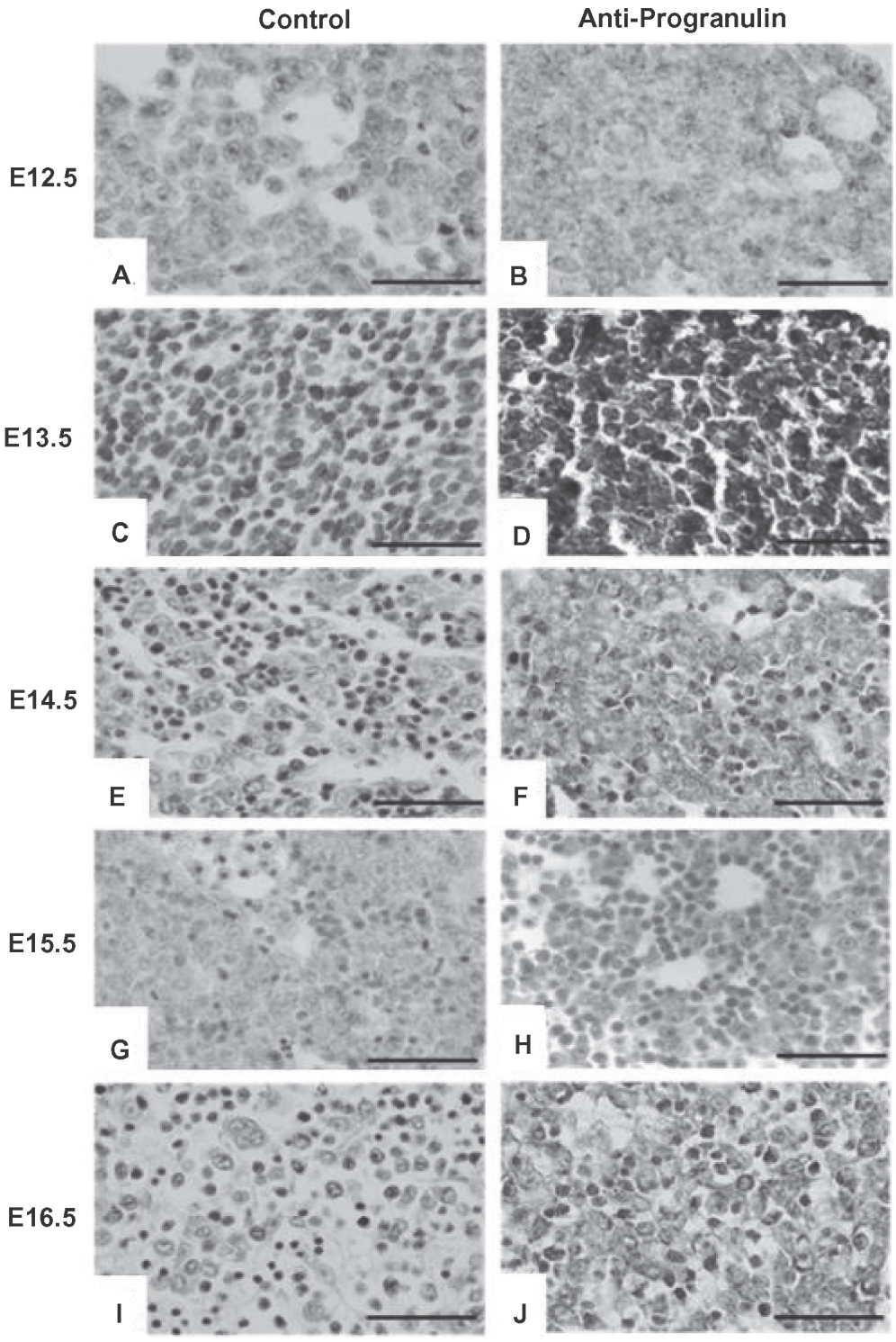

Figure 1 - Photomicrographs of immunohistochemistry + hematoxylin stain reaction for marking anti-progranulin (citoplasmatic immunereactivity) in the liver of Fischer 344 rat embryos at different gestational stages (E12.5; E13.5; E14.5; E15.5 and E16.5). Control – negative control without antibody. Observe greater anti-progranulin positivity in E13.5. Bar: 150μm

Fonte: (USP, São Paulo, 2012).

positive to marking and the peak of positivity was at E13.5 (Figure 1D).

Immunohistochemistry for marking of HNF4α

Anti-HNF4α was also positive in the liver during all the embryonic stages studied (Figure 2). However, it was highly positive during stages E12.5; E13.5 and E14.5 (Figure 2B, D and F), and low during periods E15.5 and E16.5 (Figure 2H and J). The peak of positivity for anti-HNF4α was at E13.5 (Figure 2D).

Immunohistochemistry for marking of PCNA

Anti-PCNA was positive in all the embryonic stages observed (Figure 3). PCNA had its peak of positivity in E12.5 (Figure 3A) and lowest positivity in E13.5 (Figure 3B).

Discussion

The results show primarily that PGRN is present in the liver during all the hepatogenic period studied, however, it shows less positivity in E12.5. Since the hepatogenesis threshold in rats is around E10.5 (ELMAOUHOUB, 2006) when the anterior and posterior intestine become visible, the fact that PGRN is hardly present in the first stage studied (E12.5) demonstrates that this growth factor probably is not related to the beginning of liver development. The fact that the greatest positivity for PGRN is during E13.5, may suggest that there is a direct relation between liver undifferentiated stem cells, also called bipotential or hepatoblast cells, and PGRN.

The hypothesis of this research was that PGRN stimulates cellular differentiation of hepatoblasts in hepatocytes during hepatogenesis. Corroborating this hypothesis with the result obtained, it is known that between E13.5-14.5 there is greater differentiation of hepatoblasts into hepatocytes and biliary epithelium cells (cholangiocytes) in mice (animals with embryonic development similar to that of rats) (MCLIN; ZORN, 2006). Furthermore, it is in these periods that greater morphologic cell differentiation of the liver bud occurs in rats (PASSOS, 2010). These results may suggest that PGRN acts direct or indirectly in differentiation of the hepatic cell lines.

HNF4α is necessary for differentiation and development of hepatocytes (MAEDA et al., 2006), and it was positive during all the process of hepatogenesis studied. Knowing that in rats the liver bud begins to develop at E10 (ELMAOUHOUB, 2006), and that HNF4α acts as a central regulator of hepatogenesis through the activation of the cascade of transcription factors that generate the final profile of gene expression in the mature hepatocyte (WATT; GARRISON; DUNCAN, 2003), it is really to be expected that this factor of transcription shows greater positivity during the beginning of the differentiation of hepatoblasts into hepatocytes in E12.5; E13.5 and E14.5, which happened in our study.

Utilizing the study of Chen et al. (2010) as a base that demonstrates that the expression of HNF4α controls various specific genes of the liver which are necessary to liver differentiation, as well as to specific functions do mature liver, it can be supposed that the expression of HNF4α is directly connected to the activation of the expression of PGRN, keeping in mind that the increase of the positivity of PGRN (at E13.5) during the differentiation of hepatoblasts into hepatocytes is only observed 1 day after the expression of HNF4α at E12.5, and there is hardly any positivity of PRGN in this last period.

Thus, positivity of HNF4α during the embryonic ages studied and the synchrony of its peak of positivity with that of the PRGN, reinforces the idea that the HNF4α factor of transcription is essential to differentiation and development of hepatocytes, to the activation of liver-specific genes Chen et al. (2010), as well as to the activation of PGRN.

The immunohistochemistry reaction for the marking of the PCNA protein was performed in order to verify the positivity of this protein present in the cell proliferation, since it is utilized as a marker of mitotic activity (NARYZHNY, 2008; STRZALKA;

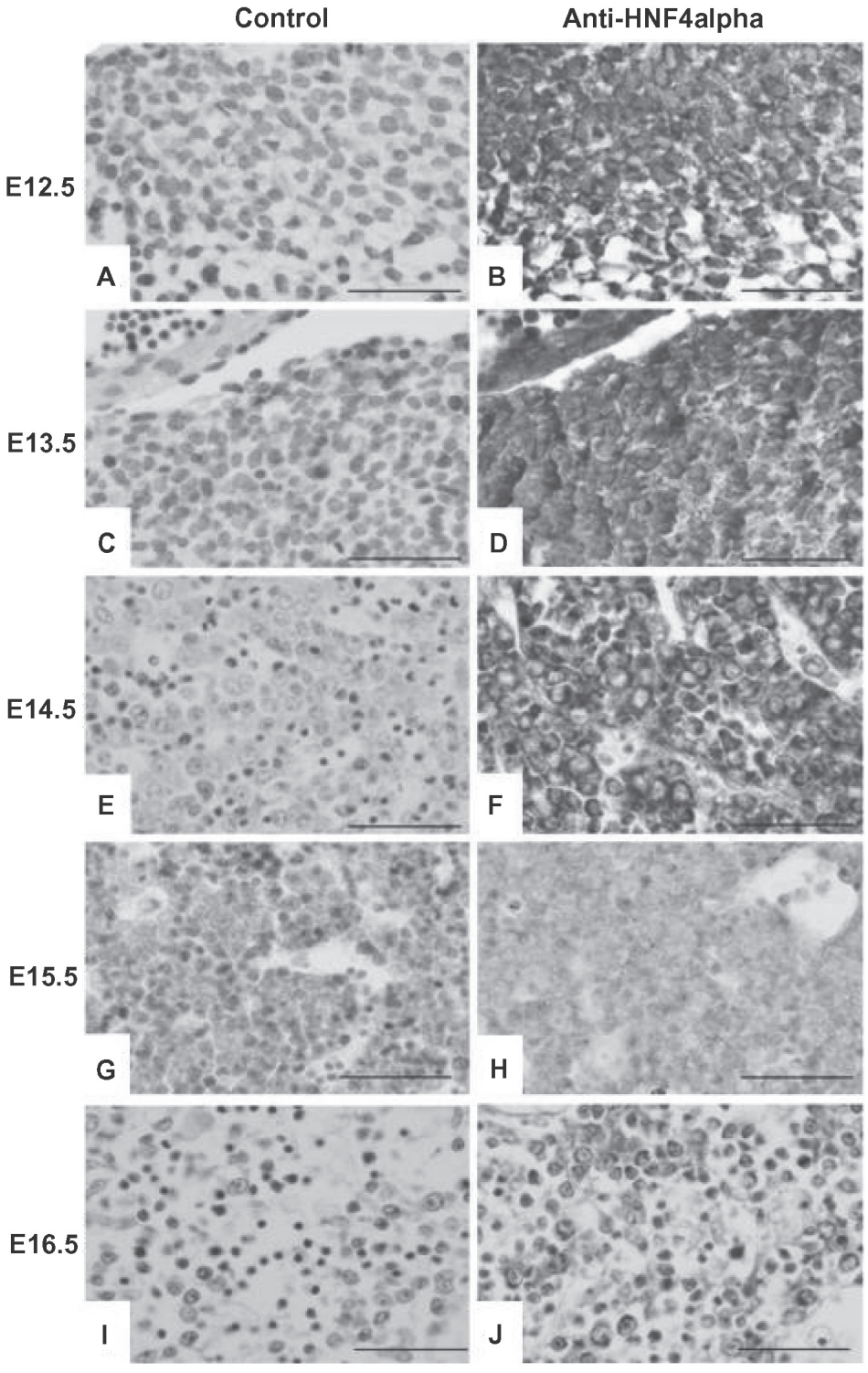

Figure 2 - Photomicrographs of immunohistochemistry + hematoxylin stain reaction for marking anti-HNF4α (citoplasmatic immunereactivity) in the liver of Fischer 344 rat embryos at different gestational stages (E12.5; E13.5; E14.5; E15.5 and E16.5). Control – negative control without antibody. Observe greater anti-HNF4α positivity between E12.5-E14.5. Bar: 150μm

Fonte: (USP, São Paulo, 2012).

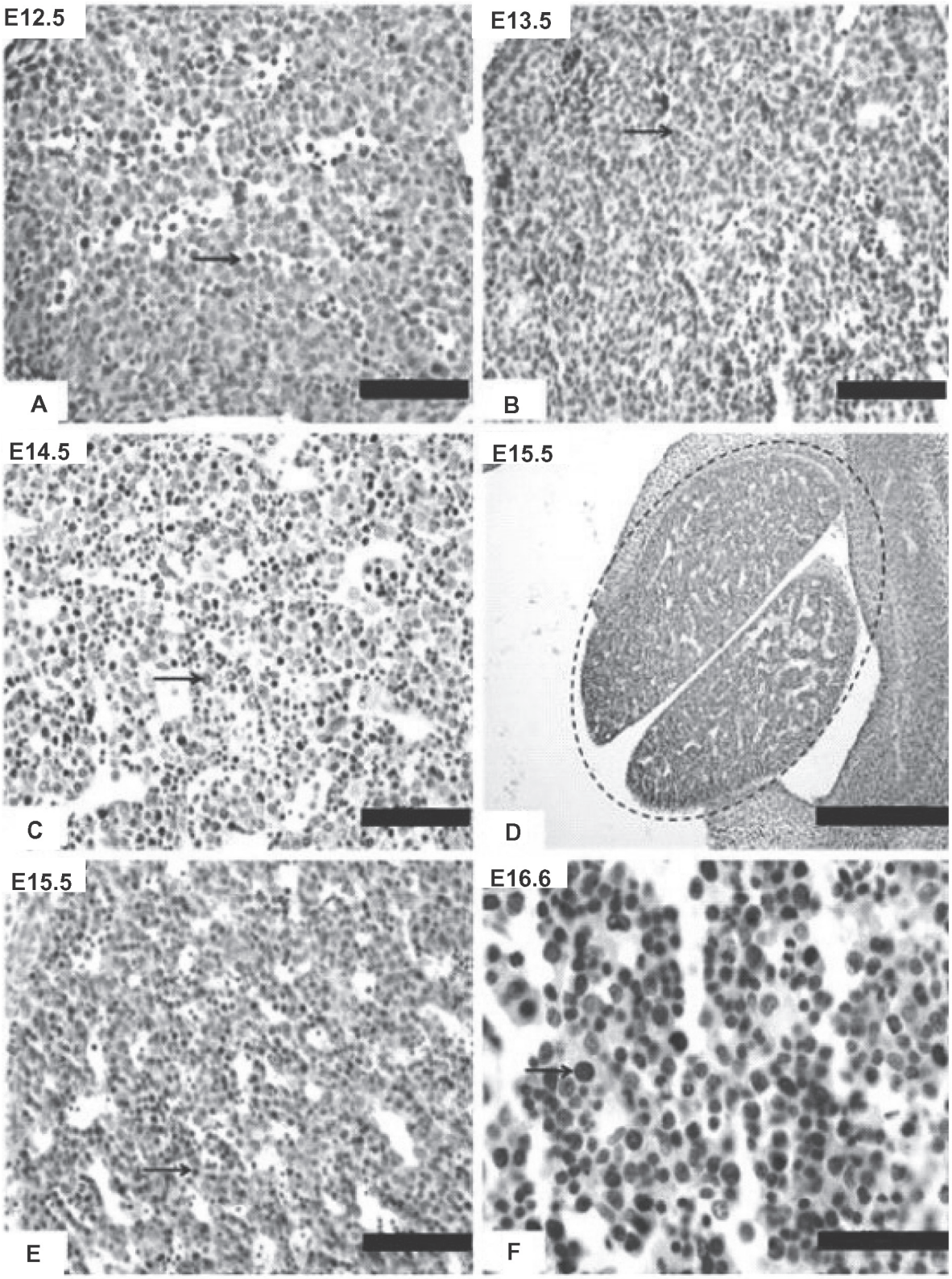

Figure 3 - Photomicrographs of immunohistochemistry reaction for PCNA (nuclear immunereactivity) + hematoxylin stain in the liver of Fischer 344 rat embryos at different gestational stages (E12.5; E13.5; E14.5; E15.5 and E16.5). The arrows represent nuclear positive marking for hepatocytes or hepatoblasts. A liver demonstrating 2 lobes identified in figure 3D. Observe greater anti-PCNA positivity in E12.5 (Figure 3A). Bar: A,B,C and E) 300µm; D) 1000µm; F) 150µm

Fonte: (USP, São Paulo, 2012).

ZIEMIENOWICZ, 2011). For this, the positivity sites of PCNA were compared to those of PGRN and HNF4α. PGRN has already been described as present in developing organs (LI et al., 1996; HE; BATEMAN, 2003), including the liver (HE; BATEMAN, 2003), and as a growth factor and inductor of cell proliferation in the process of incubation, adhesion and growth of blastocysts (DANIEL et al., 2003; QIN et al., 2005). Thus, PGRN could also be acting in proliferation, and not only in differentiation of the developing liver.

The results demonstrated that there is a peak of PCNA positivity at E12.5, and that positivity is lower at the other periods. Since the embryo is in development, with high cell proliferation, and PGRN has already been described as precursor of cell proliferation (LI et al., 1996; DANIEL et al., 2003; HE; BATEMAN, 2003; QIN et al., 2005), a peak of positivity was expected for PCNA simultaneously with the peak for HNF4α and PGRN, thus showing that PGRN has a function in cell proliferation during hepatogenesis, and this did not happen.

Thus, it seems that first there is a peak of cell proliferation at E12.5, which can be induced by HNF4α, which is highly positive from E12.5 to E14.5. Posteriorly there would be a peak of cellular differentiation at E13.5 compelled by PGRN after induction of HNF4α. Corroborating this hypothesis, both peaks of positivity for PGRN and HNF4α were at E13.5. Afterwards, (E14.5-E16.5), the levels of positivity for PGRN, HNF4α and PCNA diminished, coinciding with the period of maturation of the liver cells (PASSOS, 2010).

Conclusion

It can be concluded that PGRN is present in hepatogenesis in different embryonic stages of F344 rats, and that it seems to be involved in the process of differentiation of hepatoblasts into hepatocytes after its activation by HNF4α, however, PGRN does not seem to assume a function of cell proliferation during hepatogenesis. Thus, it would be possible to use PGRN in future protocols of liver cell differentiation aimed as cellular therapy in Regenerative Medicine.

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