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Hepatocytes Deficient in CCAAT/Enhancer Binding Protein α (C/EBPα) Exhibit both Hepatocyte and Biliary Epithelial Cell Character

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To further elucidate the role of CCAAT/Enhancer Binding Protein α (C/EBPα) in hepatocyte differentiation, we investigated fetal and newborn C/EBPα-deficient (C/EBPα −/−) mice using confocal microscopy and markers specific for hepatocyte (AFP) and biliary epithelial cell (A6) differentiation. Histologically, in fetal liver of C/EBPα −/− mice, pseudoglandular structures appeared starting at 16.5 days of gestation. In newborn livers, the diameters of these structures greatly increased. They were randomly distributed between portal and central veins and interfered with the establishment of normal hepatic plates. However, the portal bile ducts developed normally. The pseudoglandular structures were lined with small hepatocytes with round nuclei and were positive for both AFP and A6 antigens. These data show that C/EBPα −/− hepatocytes exhibit biliary epithelial cell characters and suggest an involvement of C/EBPα in the control of the switch in the differentiation of bi-potential hepatoblasts along the hepatocyte lineage.

Key Words: **knockout mice; transcription factors; development; cell differentiation; A6; hepatoblasts.**

CCAAT/Enhancer Binding Protein α (C/EBPα) is a member of the C/EBP family of leucine zipper transcription factors. As a positive transactivator, it regulates the expression of numerous genes expressed in the liver and fat tissue (1, 2, 3).

C/EBPα knockout (C/EBPα −/−) mice have recently been generated in our laboratory as well as in others (4, 5). C/EBPα −/− mice die within 10 hours after birth due to severe hypoglycemia and respiratory failure. C/EBPα −/− hepatocytes exhibit pseudoglandular structure similar to the pseudoglandular type of human hepatocellular carcinoma (5). Pseudoglandular structures of C/EBPα −/− liver have microvilli, suggesting that they have the character of biliary epithelial cells (4). Alfa-fetoprotein (AFP) expression levels are increased about 2-fold, indicating that C/EBPα −/− hepatocytes are less differentiated (5). These observations suggest that C/EBPα −/− hepatocytes have both hepatocyte and biliary epithelial cell character.

Since normal liver development is disrupted in C/EBPα −/− mice, fetal liver analysis should reveal alterations in liver development in the absence of C/EBPα expression. Liver emerges from the ventral foregut endoderm (6, 7), giving rise to hepatoblasts. Around day 16 of fetal development, the liver is highly enriched in hepatoblasts that have the potential to differentiate to hepatocytes or biliary epithelial cells as the liver develops (8, 9). At approximately the same period C/EBPα mRNA is detected (10, 11) and its expression increases on subsequent gestational days (10). These observations suggest that C/EBPα may be an important regulator in the differentiation of hepatocytes from hepatoblasts and especially important around day 16 of gestation. However, no fetal liver analysis has been done in C/EBPα −/− mice. Therefore, this study was designed to analyze fetal liver development in C/EBPα −/− mice focusing on 16.5 days of gestation. We also investigated the differentiation state of C/EBPα −/− hepatocytes at birth using markers specific for hepatocyte (AFP) and biliary epithelial cell (A6) lineages (9, 11, 12, 13). We have found that the development of hepatoblasts to hepatocytes was affected by the lack of C/EBPα starting from 16.5 days of gestation.

MATERIALS AND METHODS

Animals. C/EBPα −/− mice were established in our lab as described (5). C/EBPα heterozygotes were housed and mated in animal facilities at NIH to breed C/EBPα −/−. They were provided food and

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acidified water *ad libitum*, and maintained in a constant light-dark cycle (lights on: 7am, off: 7pm). Mice were sacrificed with carbon dioxide under NIH guidelines for proper animal procedure. Since newborn C/EBPα −/− mice die within 10 hours due to hypoglycemia and respiratory failure, they were analyzed immediately after birth (4). To genotype mice by Southern blot analysis, tail was removed from the newborn. DNA was isolated, digested, and analyzed as described (5). Mice with only wild type allele were determined as wild type (+/+), with both wild type and targeted allele as heterozygote (+/−), and with only targeted allele as knockout (−/−). Pregnancy was determined by the presence of sperm in the vaginal smear. Noon of the first day at which sperm was detected was considered as 0.5 days of gestation. Pregnant females were sacrificed on 14.5, 16.5, and 18.5 days of gestation. Heart, lung, and kidney were removed from the fetus for genotyping. Fetal liver was removed for histological examination.

For histological analysis, the excised liver was divided into 2 pieces: one was fixed in 10 % buffered formaldehyde and the other was embedded in OCT compound, snap-frozen in 2-methyl butane chilled with dry ice, and stored at −80°C. Formalin-fixed specimens were embedded in paraffin, and 4 μm sections were stained with Hematoxylin & Eosin (H&E) (American Histolab Inc., MD).

Immunohistochemistry. 5μm cryostat sections were placed on glass slides treated with poly-l-lysine, air-dried and stored at −80°C.

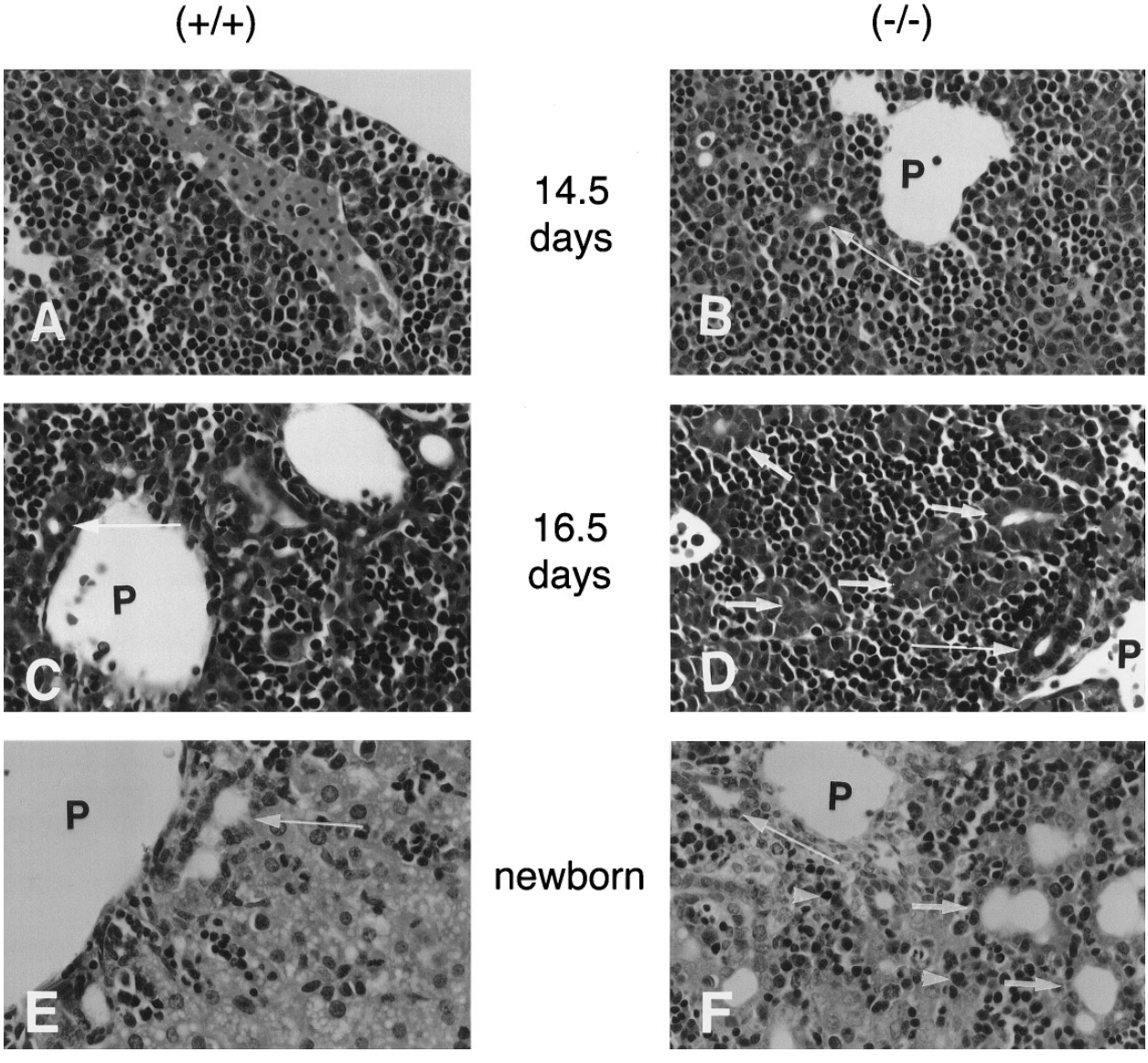

FIG. 1. Comparison of liver morphology between wild type (+/+) and C/EBPα deficient (−/−) (H&E). (A and B) Abundant hematopoietic cells were observed in both +/+ and −/−. (C) On day 16.5, in +/+ liver there was formation of bile ducts seen next to portal vein. (D) In contrast, in −/− liver, abundant pseudoglandular structures were present scattered throughout the liver. (E) In newborn +/+ liver, the formation of normal hepatic plates was observed with remaining hematopoietic cells inside the sinusoids. (F) In newborn −/− liver, note the abundant formation of pseudoglandular structures with larger diameters than seen at day 16.5 (D) and 18.5 (not shown). Left panels are from wild type mice (+/+) and right panels from C/EBP deficient (−/−). Arrows: pseudoglandular structures. Thin arrows: bile ducts. Arrow heads: hematopoietic cells. P: portal vein. Magnification: 400×.

Before staining, sections were fixed in 100% acetone at 4°C for 10 minutes and rinsed in PBS. Non-specific staining was inhibited with 5% normal goat serum (DAKO, CA) and 1% bovine serum albumin (Sigma, MO) in PBS (blocking solution) for 20 min. Sections were incubated with anti-AFP antibody (ICN Biochemicals, Inc., CA) 1:100 overnight at 4°C followed by incubation with A6 antibody (kindly provided by Dr. Engelhardt, N.V.) 1:10 overnight. Sections were incubated with goat BODIPY FL conjugated anti-rabbit IgG antibody 1:100 (Molecular Probe, OR) for one hour. Sections were incubated with goat Texas Red-X conjugated anti-rat IgG antibody 1:100 (Molecular Probe, OR) for one hour.

RESULTS

C/EBPα −/− Hepatocytes do not Form Normal Hepatic Plates in Fetal Liver

On day 14.5 of gestation, there was no difference in morphology between wild type and −/− liver. Abundant hematopoietic cells were present in both (Fig. 1 A, B). On day 16.5 of gestation, in wild type liver, the

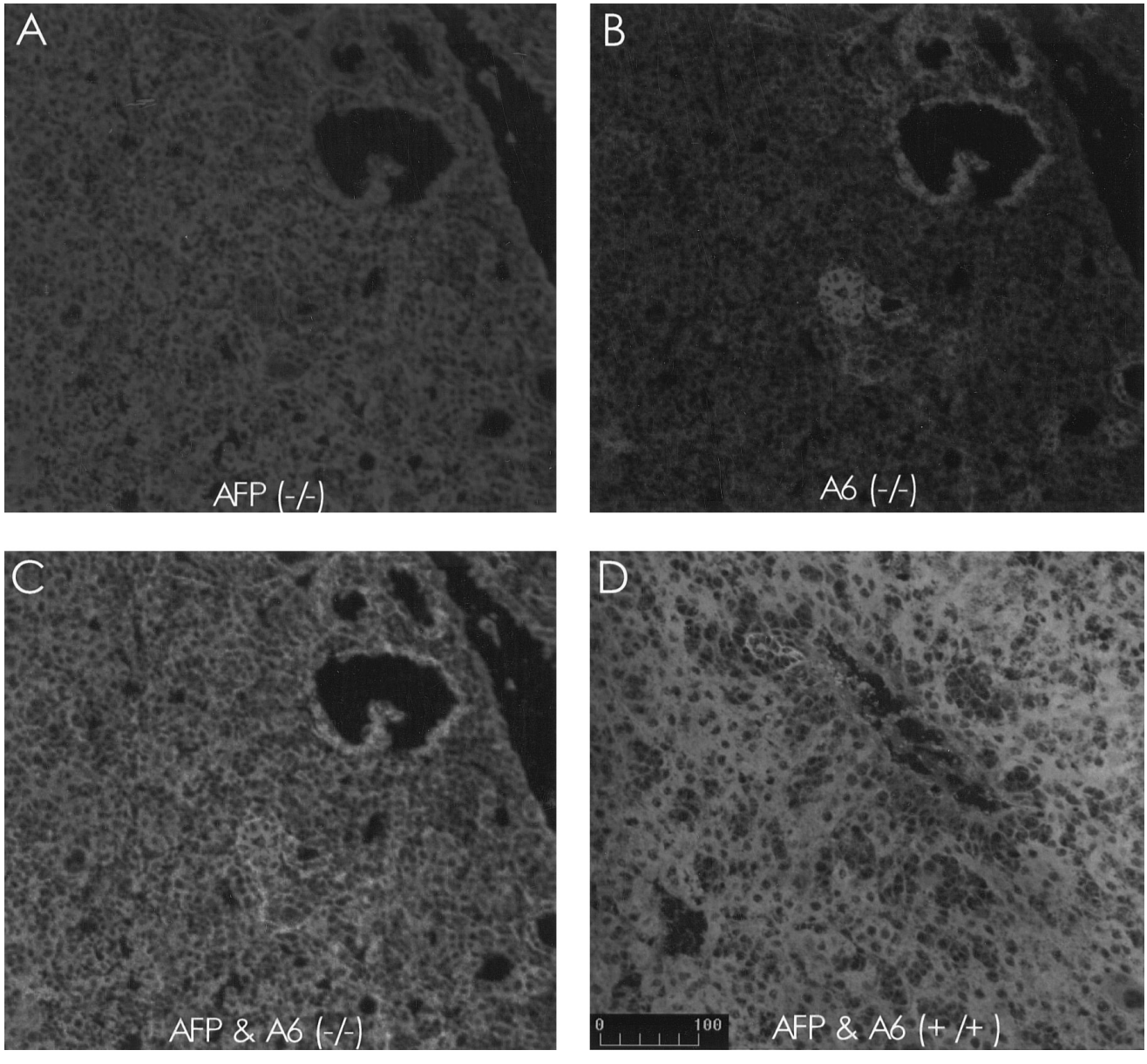

FIG. 2. Confocal analysis of newborn wild type (+/+) and C/EBPα −/− liver (−/−) with AFP and A6 double staining. (A) Hepatocytes in a pseudoglandular structures and hepatic plates were positive for AFP. (B) Positive staining in A6 was clearly seen in hepatocytes lining pseudoglandular structure. (C) Note that hepatocytes in abundant pseudoglandular structures were positive in both AFP and A6. (D) Note that biliary epithelial cells were positive in AFP and A6. A: anti-AFP-BODIPY FL of −/− liver. B: anti-A6-Texas Red −/− liver. C: merged image of A and B. D: merged image of anti-AFP-BODIPY FL and anti-A6-Texas Red of +/+ liver.

hepatoblasts increased in size and number. In contrast, in C/EBPα −/− liver, hepatoblasts formed frequent pseudoglandular structures which were randomly distributed between portal and central vein (Fig. 1 D). On day 18.5, the size of these structures increased and they dominated the hepatic plates from this time up to newborn liver. By that time, pseudoglandular structures had greatly dilated lumens and were lined with lightly stained hepatocytes having round nuclei (Fig. 1 F). However, the formation of bile ducts was not affected in these knockout mice. Figure 1 F demonstrates the presence of typical bile duct structure in the portal area lined with small cells containing dark stained oval nuclei.

C/EBPα −/− Hepatocytes Lining Pseudoglandular Structures Exhibited Both Hepatocyte and Biliary Epithelial Cell Antigen Markers

In order to clarify the origin of pseudoglandular structures characteristic of the early stage of development in these mice, we used confocal microscopy and antigen markers specific for hepatocyte (AFP) and biliary epithelial cell (A6) lineages. In newborn livers of wild type mice, all hepatocytes demonstrated homogenous cytoplasmic staining for AFP (Fig. 2D). Cells lining bile ducts as well as singular cells located at the edge of the hepatic plates were strongly positive for A6 consistent with the previous observations (13). Also, in newborn livers, cells lining the developing bile ducts were positive for AFP (Fig. 2D). In newborn livers of C/EBPα −/− mice, all hepatocytes expressed AFP whether in hepatic plates or pseudoglandular structures (Fig. 2A). In addition, cells lining pseudoglandular structures randomly distributed throughout the parenchyma were strongly A6-positive (Fig. 2B). Thus, using confocal microscopy, we have demonstrated that the pseudoglandular structures developing in C/EBPα −/− livers co-expressed antigens specific for hepatocyte and biliary epithelial cell lineages (Fig. 2C).

DISCUSSION

In liver development, hepatoblasts appear near vascular spaces with the potential to differentiate into both hepatocytes and biliary epithelial cells from 9 to 13 days of gestation (9, 14). Precursors of intrahepatic bile ducts develop around portal vein at 13.5 days of gestation (14), and the first bile ducts at 15 days of gestation (9). C/EBPα has been implicated as an important molecule for proper liver development (15). Previously it has been shown that disruption of C/EBPα gene results in abnormal liver architecture at birth (4, 5), but no report exists on the fetal liver analysis of C/EBPα −/− mice. Here we show that normal formation of hepatic plates was disrupted by abundant formation of pseudoglandular structures starting from 16.5 days of gestation. On the other hand, it seems likely that formation of the bile duct is not affected.

Our results suggest that pseudoglandular structures abundant in developing C/EBPα livers have the properties of both biliary epithelial cells and hepatocytes because they exhibit the presence of both AFP and A6 antigens. However, C/EBPα −/− hepatocyte forming plates loose the marker of biliary epithelial cells. These data are consistent with the previous observations that C/EBPα −/− hepatocytes are positive for AFP and form duct like structures when transplanted into mice (16). The coexpression of antigens specific for hepatocytes and biliary epithelial cells is the properties of hepatoblasts (9, 14, 17). Our data show that a large proportion of hepatocytes in new born C/EBPα −/− livers retain the markers of biliary epithelial cells, and thus exhibit the characteristics of hepatoblasts (17) or immature hepatocytes (18). Furthermore, the presence of pseudoglandular structures which have the antigen profile of bi-potential precursors in newborn C/EBPα −/− livers demonstrate a severe disruption of normal hepatocyte differentiation.

It has been reported previously that mRNA expression of C/EBPα appears on day 16 or 17, and increases on subsequent gestational days (10, 11). In C/EBPα −/− mice, we have found that pseudoglandular structures developed at the same time. Taken together, the results suggest a critical role of C/EBPα in differentiation of hepatoblasts between 16 and 17 days of liver development.

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REFERENCES

1. Costa, R. H., Grayson, D. R., and Darnell, J. E., Jr. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3840.
2. Xanthopoulos, K. G., Mirkovich, J., Decker, T., Kuo, C. F., and Darnell, J. E., Jr. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4117.
3. Herrear, R., Ro, H. S., Robinson, G. S., Xanthopoulos, K. G., and Spiegelman, B. M. (1989) *Mol. Cell Biol.* **9,** 533.
4. Wang, N. D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V, Wilde, M. D., Taylor, L. R. et al. (1995) *Science* **269,** 1108–1112.
5. Flodby, P., Barlow, C., Kylefjord, H., Ährlund-Tichter, L., and Xanthopoulos, K. G. (1996) *J. Biol. Chem.* **271,** 24753–24760.
6. Du Bois, A. M. (1995) *in* The Liver, Morphology, Biochemistry, Physiology. Volume 1. (Rouiller, C., Ed.), pp. 1–39, Academic Press, New York.

7. Wilson, J. W., Groat, C. S., and Leduc, E. H. (1963) *Ann. NY Acad. Sci.* **111,** 8–24.
8. Houssaint, E. (1980) *Cell Differ.* **9,** 269–279.
9. Shiojiri, N., Lemire, J. M., and Fausto, N. (1991) *Cancer Res.* **51,** 2611–2620.
10. Kuo, C. F., Xanthopoulo,s K. G., and Darnell, J. E., Jr. (1990) *Development* **109,** 473–481.
11. Nagy, P., Bisgaard, H. C., and Thorgeirsson, S. S. (1994) *J. Cell Biol.* **126,** 223–233.
12. Engelhardt, N. V., Factor, V. M., Yasova, A. K., Poltoranina, V. S., Baranov, V. N., and Lasareva, M. N. (1990) *Differentiation* **45,** 29–37.
13. Engelhardt, N. V., Factor, V. M., Medvinsky, A. L., Baranov, V. N., Lazareva, M. N., and Poltranina, V. S. (1993) *Differentiation* **55,** 19–26.
14. Shiojiri, N., and Nagai, Y. (1992) *Anat. Embryolo.* **185,** 17–24.
15. Debeva, M. D., Hurston, E., and Sharitz, D. A. (1995) *Am. J. Pathol.* **147,** 1633–1648.
16. Soriano, H. E., Kang, D. C., Finegold, M. J., Kicks, M. J., Wang, N. D., Harrison, W., and Darlington, G. J. (1998) *Hepatology* **27,** 392–401.
17. Rogler, L. E. (1997) *Am. J. Pathol.* **150,** 591–602.
18. Shiojiri, N., and Mizuno, T. (1993) *Anat. Embryolo.* **187,** 221–229.