Contents lists available at ScienceDirect

IF: 0.926

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi: 10.1016/S1995-7645(14)60353-9

BMP-4 induced proliferation and oriented differentiation of rat hepatic oval cells into hepatocytes

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ARTICLE INFO

Article history:
Received 15 February 2015
Received in revised form 20 March 2015
Accepted 15 April 2015
Available online 20 May 2015

Keywords:
Bone morphogenetic protein-4
Transforming growth factor- β
Hepatic progenitor cells
Proliferation
Differentiation

ABSTRACT

Objective: To explore the role of bone morphogenetic protein 4 (BMP-4) in hepatic progenitor cells (HPCs). **Methods:** The effect of BMP-4 on rat hepatic oval cells was examined by using the WB-F344 rat hepatocytic epithelial stem-cell-like cell line. This hepatocytic cell line could exert various hepatocyte functions including the secretion of albumin and urea. Immunohistochemistry was used to examine the effects of BMP-4 and its antagonist, Noggin, on the proliferation and differentiation of these cells, cellular uptake and excretion of indocyanine green, the periodic acid-schiff (PAS) assay for glycogen storage and the expression of hepatic markers. **Results:** Our results showed for the first time that BMP-4 may acted as a potential inducer of hepatic differentiation in rat hepatic oval cells. **Conclusions:** This cell source offers a much-needed attractive and expandable source for future investigations of drug screening, stem cell technologies and cellular transplantation, in a society with increasing levels of liver disease and damage.

1. Introduction

The liver performs many complex functions including carbohydrate metabolism, urea and lipid metabolism, storage of essential nutrients and the production and secretion of bile acids, into which metabolites of drugs and other compounds are excreted[1]. Liver dysfunction is a major health problem across the world, yet human donor livers are in short supply. An unlimited supply of functional hepatocytes from other sources is therefore of great interest in terms of cell engineering for clinical purposes[2]. Embryonic stem cells (ESCs)[3], bone marrow stem cells[4], liver stem cells/oval cells[5], cord blood cells[6] and fetal hepatocytes[7] are all cell types that display the potential to develop into viable hepatocytes. However, the conditions necessary to direct the differentiation of human stem cells into a specific lineage, such as hepatocytes, are not yet fully defined. The transforming growth factor-beta (TGF- β) superfamily of proteins is important extracellular signaling proteins participating in many developmental and physiological processes. Bone morphogenetic protein-4 (BMP-4), a member of the TGF- β superfamily, was originally identified as a bone- and cartilage-inducing factor and has critical roles in vertebrate development[8,9]. Liver stem cell activation was first described in the rat, where the combination of chemical damage and partial hepatectomy caused the emergence of a new cell type described according to its nuclear morphology as an oval cell[10]. These cells are thought to be bipotential progenitors capable of yielding both hepatocytes and bile ducts. Consequently, oval cells are hypothesized to be the progeny of intrahepatic stem cells, also referred to as adult liver stem cells[11]. An understanding of the mechanisms by which these cells are activated to proliferate and differentiate during regeneration is important for the development of new therapies to treat liver disease. Oval cell activation is the first step in progenitor-dependent liver regeneration in response to certain types of injury[10].

Several studies[11-13] have reported the induction of ESCs differentiation into hepatic cells both *in vivo* and *in vitro*. The *in vitro*

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generation of ESC-derived hepatic cells has also been reported using BMP-4. However, these studies show the capabilities of multipotent progenitor cells, which may differ from the more readily available oval cell. In this study, we attempted to understand how the liver repairs itself following surgical or drug-induced liver injury. This was carried out by examining the differentiation of rat oval cells into hepatic cells using BMP-4 in the presence and absence of its antagonist, Noggin[14].

2. Materials and methods

2.1. Culture of hepatic oval cells

WB-F344 cells were kindly provided by Dr. Hong shen (Central South University, Xiangya Hospital)[15]. Hepatic oval cells were prepared and cultured in DMEM/Ham's F12 (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 0.5 U/mL insulin, 1 ng/mL epidermal growth factor (EGF, PeProTech, Rehovot, Israel), 0.5 ng/mL stem cell factor (SCF, PeProTech), 100 U/mL penicillin and 100 μg/mL streptomycin. The medium was replaced every 4–5 days. After the cells formed clones those with a cobblestone appearance were isolated and cultured in a separate dish. Thereafter, the medium was changed every 3–4 days and the cells were passaged when they formed a near-confluent culture[16].

The WB-F344 cells were divided into four groups which were exposed to different culture conditions. These groups were: a control group (W group) that did not undergo any treatment; BMP-4 group (B group) in which the oval cells were cultured with BMP-4 (50 ng/mL); Noggin group (N group) in which the oval cells were cultured with Noggin (200 ng/mL); BMP-4 and Noggin group (N+B group) in which the oval cells were cultured with BMP-4 (50 ng/mL) and Noggin (200 ng/mL). For the purposes of this study, all four groups were cultured concurrently for 2 months. We analyzed the growth, morphology and hepatocyte function across all four groups throughout the 2 months of culture. Following 2 months in culture the cells in the B group showed expression of hepatic markers, thus this time point was chosen to compare all cell lines for hepatocyte function.

2.2. ELISA

The oval cells were differentiated into hepatocytes as described. Culture supernatants were incubated for 24 hr after fresh medium was added prior to their collection for analysis by ELISA of the amount of ALB secretion. ELISA kits for ALB were purchased from Uscn Life Science Inc., ELISAs were performed according to the manufacturer's instructions. The amount of ALB secretion was calculated according to each standard followed by normalization to the protein content per well.

2.3. MTT & cell counting

Cells from each of the four groups were plated in 96 well plates in triplicate to minimize the variability of the results. The cells were analyzed on days 2, 4 and 6 of culture. Following the appropriate incubation time 1:10 volume of MTT solution to culture solution was added (5 mg/mL) up to a total volume of 15 μL in a flat bottom 96-well plate. The solutions were mixed by shaking on a plate shaker for 5 min. The plates were further incubated for 6 h at 37 ℃ in a 5% CO_2 incubator. 150 μL of acidified isopropanol was added to each well and resuspended until all crystals had been dissolved. The optical density was measured at 540 and 720 nm to allow for the correction of background noise.

2.4. Urea secretion

The oval cells were differentiated into hepatocytes as described. The culture supernatants, which were incubated for 24 hr following the addition of fresh medium, were collected and analyzed for the amount of urea secretion that had occurred. Urea measurement kits were purchased from BioAssay Systems (Hayward, USA). The experiment was performed according to the manufacturer's instructions. The amount of urea secretion was calculated according to each standard followed by normalization to the protein content per well.

2.5. Cellular uptake and excretion of indocyanine green

Indocyanine Green (ICG) (Sigma-Aldrich, St Louis, MO, USA) was dissolved in DMSO at 100 mg/mL, then added to a culture medium of the oval cells to a final concentration of 1 mg/mL. After incubation at 37 ℃ for 60 min, the medium with ICG was discarded and the cells were washed with PBS. The cellular uptake of ICG was then examined by microscopy. PBS was replaced by the culture medium and the cells were incubated at 37 ℃ for 6 hr. The excretion of ICG was examined again by microscopy.

2.6. Immunohistochemistry

The cells were fixed with methanol or 4% paraformaldehyde (Wako, Saitama, Japan). After blocking with PBS containing 2% BSA (Sigma-Aldrich, St Louis, MO, USA) and 0.2% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA), the cells were incubated with primary antibody at 4 ℃ for 16 hr, followed by incubation with a secondary antibody at room temperature for 1 hr.

2.7. Periodic acid–schiff (PAS) assay for glycogen

The cells were fixed with 4% paraformaldehyde and stained using a PAS staining system (Sigma-Aldrich, St Louis, MO, USA) of differentiation according to the manufacturer's instructions.

2.8. Transmission electron microscopy

Human oval cell-derived hepatic cells were fixed with 2% glutaraldehyde cacodylate buffer for 30 min and post-fixed with 1% osmium tetroxide for 30 min, after which they were dehydrated through a series of graded ethanol. Samples were embedded in Epok 812 (Oken Trading, Tokyo, Japan), and ultrathin sections were cut with an ultramicrotome (MT7000-Ultra; RMC, Tucson, AZ). Samples were stained with uranyl acetate and lead citrate and were then viewed with an electron microscope (H-7500; Hitachi, Tokyo, Japan).

2.9. Preparation of cell extracts and western blotting analysis

Cells were washed with phosphate-buffered saline (PBS) and collected in 1 mL of lysate buffer (50 mM Tris-HCl pH 7.3, 150 mM NaCl, 3 mM MgCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 1.0% Triton X-100, Sigma-Aldrich, St Louis, MO, USA) supplemented with protease and phosphatase inhibitors. The extraction supernatant was collected and 30 μg of protein from each sample was resolved on 4%–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred, and immunoblotted onto nitrocellulose membrane. Anti-ALB (ABGENT, San Diego, CA, USA), anti-Cx43 (CST, Boston, MA, USA) and anti-β-actin antibodies (Sigma-Aldrich, St Louis, MO, USA) were used as primary antibodies.

3. Results

This experiment studied differentiation assessing both the cell morphology and function. The functional analyses included albumen and urea secretion, cell viability, drug metabolism, the accumulation of glycogen, and hepatic marker expression. Changes in cellular morphology were observed using transmission election microscopy. The level of urea secreted from each of the four different differentiation conditions was analyzed weekly during the course of the experiments. The data illustrate that the amount of urea secreted from cells treated with BMP-4 (B group) increased gradually over the period of incubation (Figure 1b) and was consistently greater than that observed in the alternative control culture conditions. The level of ALB secretion observed by the B group was also significantly higher than seen under any of the other differentiation conditions (Figure 1a). This was supported by an increase in albumin expression in cells treated with BMP-4 alone compared to control-treated cultures as assessed by both immunofluorescence (Figure 2a) and western blot analysis (Figure 2d). The secretion of both albumin and urea are markers of the differentiation of hepatocytes. Thus the secretion of these markers by rat oval cells cultured with BMP-4 indicates that the rat hepatic oval cells, WB-F344, may be able to differentiate down the hepatic lineage in the presence of BMP-4, and suggests that this growth factor may operate within hepatic tissues.

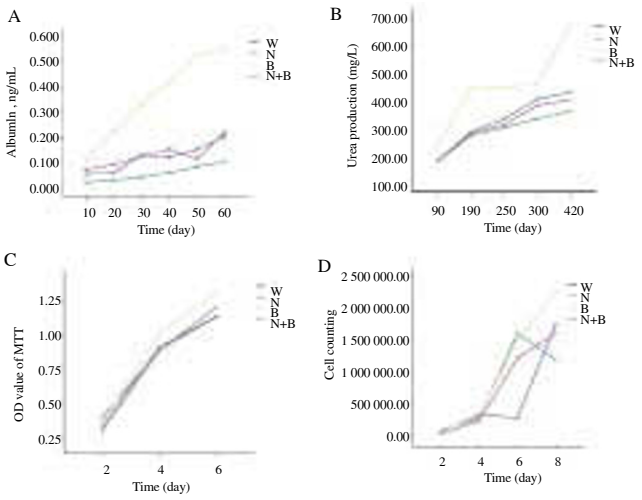

Figure 1. Increase in hepatocyte function of rat hepatic oval cells under different culture conditions.

(A) The amount of ALB secretion was examined by ELISA. (B) The amount of urea secretion was examined across the 4 culture conditions. (C, D) MTT & cell counting.

Cells cultured with BMP-4 as part of the B-group exhibited expression of the hepatocyte marker, G6 by immunofluorescent analysis (Figure 2b) and an abundant storage of glycogen as determined by the PAS assay (Figure 2c). Some G6 and glycogen staining was also observed in the control-treated cultures, though not to the same levels as with BMP-4 alone. Moreover, the expression of connexin-43, an endodermal marker was seen to decrease in WB-F344 cultures treated with BMP-4, compared to those treated with noggin or untreated, as determined by western blot. This staining indicates that the presence of BMP-4 within the culture medium promotes the differentiation of WB-F344 cells to hepatocytes.

The oval cell-derived hepatic function of the cultures was further evaluated by examining the cells' uptake of Idocyanine Green (ICG) (Figure 3a). B group cells, cultured with BMP-4, had the ability to uptake ICG and to excrete ICG in a culture without ICG for 6 h. These results suggest that oval cell-derived hepatocyte-like cells express the necessary conjugating enzyme activity to metabolize various drugs, potentially making them useful in drug screening analyses.

Transmission electron microscopy images of the treated cell cultures suggested that B group cells, treated with BMP-4 possess the characteristics of mature hepatocytes (Figure 3b), supporting the hypothesis that the presence of BMP-4 may induce the differentiation of rat hepatic oval cells along the hepatocyte lineage.

Taken together these results suggest that rat hepatic oval cells can be induced by BMP-4 to differentiate into hepatocyte-like cells.

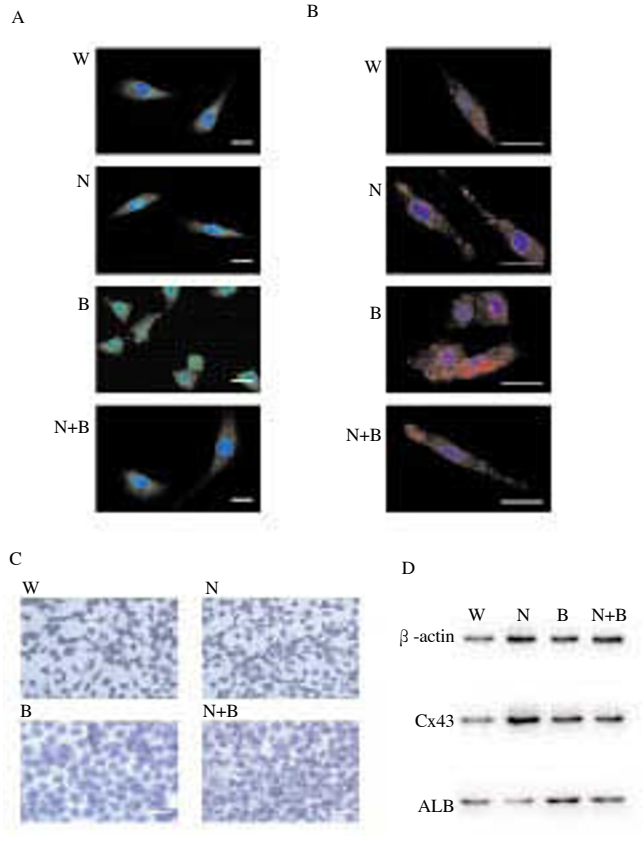

Figure 2. Differentiation of rat oval cells into hepatic lineages.

(A, B) Immunohistochemistry shows expression of the hepatocyte markers ALB (green) and G6 (red). Nuclei were counterstained with DAPI (blue). Bars indicate 20 μm. (C) Periodic acid-Schiff (PAS) assay for glycogen staining carried out on same day differentiated oval cells indicates numerous hepatocytes within the colonies with cytoplasmic glycogen storage (dark red). Bars indicate 40 μm. (D) Western blot analysis of ALB and Connexin-43 expression under al 4 culture conditions.

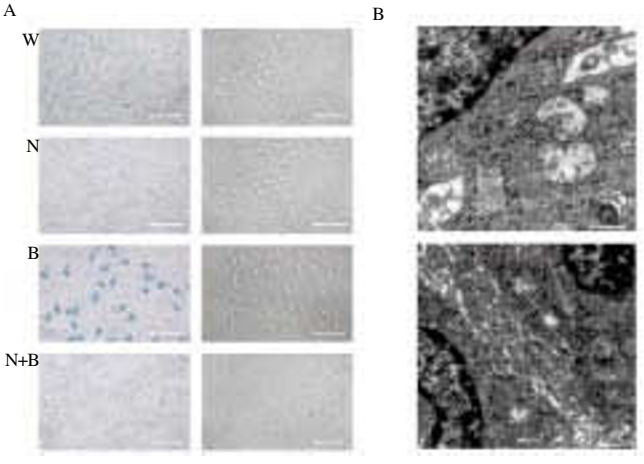

Figure 3. Analyses of rat oval cell-derived hepatocytes.

(A) Cells cultured under 4 different conditions were examined for their ability to take up ICG (left) and release it 6h thereafter (right). Bars indicate 40 μm. (B) Transmission electron microscopy of oval cell-derived differentiated cells. Bc, bile canaliculus; Mi, microvillus; arrows represent the junctional complexes between cells; bars indicate 6 μm

4. Discussion

The differentiation of progenitor cells into various cell types is key to cell engineering and important in the repair of injury and regeneration of organs[17]. In response to injury, hepatocytes themselves undergo activation to proliferate and differentiate into parenchymal cells, thus these processes show good therapeutic potential. However, oval cells may contribute to newly formed hepatocytes and cholangiocytes[18], but very little is known about the biology of oval cells and the culture conditions suitable to promote oval cell differentiation and expansion in vitro.

Examining the four different culture conditions together we found that the rat hepatic oval cells incubated with BMP-4 alone can differentiate into hepatocyte-like cells, though this is a slow process taking nearly 2 months in culture. Cultures containing both BMP-4 and noggin and the untreated cultures also showed signs of differentiation along the hepatic lineage, though to a lesser extent than the cells treated with BMP-4. Cultures treated with noggin alone showed no signs of differentiation. The differentiation markers seen in cultures treated with both BMP-4 and noggin demonstrate that the concentration of noggin used in this study was not sufficient to neutralize the effects of BMP-4, thus higher concentrations of this BMP antagonist should be used to fully block BMP signaling in vitro in future studies. Additionally, the differentiation markers observed in untreated cultures may indicate spontaneous differentiation in vitro or that some components of the media can possibly promote the differentiation of rat oval cells, the effects of which may be blocked by the presence of Noggin.

Using electron microscopy to study the cellular ultrastructure, we showed that the morphological characteristics of the cells treated with BMP-4 changed slowly during the two month experimental period. Cell volume increased and the cell shape changed from a typical spindle epithelial morphology to a polygonal shape more associated with hepatocytes. Microvilli appeared on the cells during the culture period, with the development of clear polarization of the cells, showing well-developed microvilli as prominent on one surface of the cell, with junctional complexes on the lateral side of the cell. These results indicate that oval cell-derived hepatic cells show normal hepatocyte ultrastructure.

To determine functional changes we analyzed the synthesis of hepatocyte markers, cell secretion and metabolism. Our results suggest that the in vitro generation of oval cell-derived hepatic cells requires BMP4, whilst Noggin is able to completely stop cell differentiation.

The mature hepatocyte markers of the secretion of Albumin (ALB) and urea, were detected and increased substantially in BMP-4 treated cells. Most cells were PAS-positive, demonstrating glycogen deposition. Additionally, the results of PAS staining, Western Blot, and transmission electron microscopy suggest that oval cell-derived

cells have the characteristics of mature hepatocytes.

With increasing levels of liver damage being seen in clinic as patients engage in more harmful diets and drug use, the regeneration of liver tissue is set to be key in not only repairing injury but also maintaining a healthy population. This study shows for the first time the potential to use hepatic oval cells to generate hepatocytes, the further investigation of which may open new doors in tissue engineering and the maintenance of quality-of-life for many patients.

Acknowledgements

This research was approved by the ethics committee of Xiangya Hospital, Central South University.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Shiraki N, Umeda K, Sakashita N, Takeya M, Kume K, Kume S. Differentiation of mouse and human embryonic stem cells into hepatic lineages. *Genes Cells* 2008; **13**(7): 731-746.

[2] Duan Y, Catana A, Meng Y, Yamamoto N, He S, Gupta S, et al. Differentiation and enrichment of hepatocyte-like cells from human embryonic stem cells in vitro and in vivo. *Stem Cells* 2007; **25**(12): 3058-3068.

[3] Hamazaki T, Iiboshi Y, Oka M, Papst PJ, Meacham AM, Zon LI, et al. Hepatic maturation in differentiating embryonic stem cells *in vitro*. *FEBS Lett* 2001; **497**(1): 15-19.

[4] Schwartz RE, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J Clin Invest* 2002; **109**(10): 1291-1302.

[5] Gupta S, LaBrecque DR, Shafritz DA. Mitogenic effects of hepatic stimulator substance on cultured nonparenchymal liver epithelial cells. *Hepatology* 1992; **15**(3): 485-491.

[6] Newsome PN, Johannessen I, Boyle S, Dalakas E, McAulay KA, Samuel K, et al. Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. *Gastroenterology* 2003; **124**(7): 1891-1900.

[7] Malhi H, Irani AN, Gagandeep S, Gupta S. Isolation of human progenitor liver epithelial cells with extensive replication capacity and differentiation into mature hepatocytes. *J Cell Sci* 2002; **115**(Pt 13): 2679-2688.

[8] Shinozuka H, Lombardi B, Sell S, Iammarino RM. Early histological and functional alterations of ethionine liver carcinogenesis in rats fed a choline-deficient diet. *Cancer Res* 1978; **38**(4): 1092-1098.

[9] Church RH, Krishnakumar A, Urbanek A, Geschwindner S, Meneely J, Bianchi A, et al. Gremlin1 preferentially binds to bone morphogenetic protein-2 (BMP-2) and BMP-4 over BMP-7. *Biochem J* 2015; **466**(1): 55-68.

[10] Yovchev MI, Grozdanov PN, Joseph B, Gupta S, Dabeva MD. Novel hepatic progenitor cell surface markers in the adult rat liver. *Hepatology* 2007; **45**(1): 139-149.

[11] Takayama K, Inamura M, Kawabata K, Sugawara M, Kikuchi K, Higuchi M, et al. Generation of metabolically functioning hepatocytes from human pluripotent stem cells by FOXA2 and HNF1alpha transduction. *J Hepatol* 2012; **57**(3): 628-636.

[12] Takayama K, Inamura M, Kawabata K, Tashiro K, Katayama K, Sakurai F, et al. Efficient and directive generation of two distinct endoderm lineages from human ESCs and iPSCs by differentiation stage-specific SOX17 transduction. *PLoS One* 2011; **6**(7): e21780.

[13] Takayama K, Inamura M, Kawabata K, Katayama K, Higuchi M, Tashiro K, et al. Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4alpha transduction. *Mol Ther* 2012; **20**(1): 127-137.

[14] Pregizer SK, Mortlock DP. Dynamics and cellular localization of bmp2, bmp4, and noggin transcription in the postnatal mouse skeleton. *J Bone Miner Res* 2015; **30**(1): 64-70.

[15] Fan J, Shen H, Dai Q, Minuk GY, Burzynski FJ, Gong Y. Bone morphogenetic protein-4 induced rat hepatic progenitor cell (WB-F344 cell) differentiation toward hepatocyte lineage. *J Cell Physiol* 2009; **220**(1): 72-81.

[16] Wang P, Cong M, Liu TH, Yang AT, Cong R, Wu P, et al. Primary isolated hepatic oval cells maintain progenitor cell phenotypes after two-year prolonged cultivation. *J Hepatol* 2010; **53**(5): 863-871.

[17] Forbes SJ, Alison MR. Regenerative medicine. Knocking on the door to successful hepatocyte transplantation. *Nat Rev Gastroenterol Hepatol* 2014; **11**(5): 277-278.

[18] Dezso K, Papp V, Bugyik E, Hegyesi H, Safrany G, Bodor C, et al. Structural analysis of oval-cell-mediated liver regeneration in rats. *Hepatology* 2012; **56**(4): 1457-1467.