Biochemical and Biophysical Research Communications 478 (2016) 1575–1581

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Downregulation of IGF-1 receptor occurs after hepatic linage commitment during hepatocyte differentiation from human embryonic stem cells

Ahmed Waraky [a, *], Eiman Aleem [a, b, c, d], Olle Larsson [a]

[a] Department of Oncology-Pathology, Cancer Center Karolinska, Karolinska Institutet, Stockholm, Sweden
[b] Institute of Molecular Medicine at Phoenix Children's Hospital, Phoenix, AZ, USA
[c] University of Arizona College of Medicine-Phoenix, Department of Child Health, Phoenix, AZ, USA
[d] Alexandria University, Faculty of Science, Department of Zoology, Alexandria, Egypt

ARTICLE INFO

Article history:
Received 25 August 2016
Accepted 27 August 2016
Available online 31 August 2016

Keywords:
IGF-1R
Hepatocyte
Differentiation

ABSTRACT

The insulin-like growth factor 1 receptor (IGF-1R) has been suggested to be involved in hepatocyte differentiation. Human hepatocyte cancer cells and stem cells are known to express IGF-1R whereas normal hepatocytes do not. In the present study we optimized a differentiation protocol and verified the different stages by established markers. The expression levels of IGF-1R and major downstream signaling proteins during differentiation from human embryonic stem cells (hESC) to mature hepatocytes were investigated. We could only demonstrate a minor decrease in IGF-1R expression during endodermal differentiation compared to hESC, but declined substantially (>50%) after hepatic lineage commitment during the hepatocyte specification and maturation stages. This downregulation was paralleled by an upregulation of ERK 1/2, AKT and insulin substrate-1. Neither inhibition nor activation of IGF-1R had any essential effect on endoderm differentiation of human embryonic stem cells. Therefore, our data suggest that IGF-1R downregulation may have a regulatory impact after initiation of hepatic lineage commitment.

1. Introduction

The insulin-like growth factor 1 receptor (IGF-1R) consists of two ligand-binding, extracellular α-subunits and two β-subunits. Ligand binding induces autophosphorylation and activation of the receptor, followed by recruitment of specific docking proteins, including Shc and insulin-receptor substrate-1 (IRS1). This leads to subsequent activation of downstream signaling pathway that include phosphatidyl inositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/ERK pathways [1–3]. IGF-1R has been indicated to play an important role in development and differentiation [4]. IGF-1R knockouts are embryonically lethal and embryos exhibit developmental delays in ossification, epidermis, muscles and central nervous system [5]. In addition, roles for IGF-1R in inducing differentiation in hematopoietic cells [6] and hepatocyte differentiation from mesenchymal stem cells [7] have been reported. During hepatocyte differentiation IGF-1R expression is downregulated, hESC have been shown to express high levels of IGF-1R [8], while normal hepatocytes (nHeps) do not express any detectable levels of IGF-1R mRNA or protein [9,10].

In the present study we investigated the expression pattern of the IGF-1R and major downstream signaling proteins during differentiation of hESCs to mature hepatocytes. To our knowledge this is the first study showing that downregulation of IGF-1R occurs after hepatic linage commitment during the hepatocyte specification and maturation stages of differentiation.

2. Materials and methods

2.1. Cell culture and reagents

H1 (WA01) hESC line was obtained from WiCell Research Institute Inc. (Madison, WI). Undifferentiated hESCs were maintained on mouse embryonic fibroblasts (SNL feeder cells) obtained from (Professor Klas Wiman, Karolinska Institutet, Stockholm, Sweden) in 5% CO_2. Before seeding the hESC, mouse embryonic SNL

* Corresponding author.
E-mail address: ahmed.waraky@ki.se (A. Waraky).

http://dx.doi.org/10.1016/j.bbrc.2016.08.157

feeder cells were treated first with mitomycin C (Sigma-Aldrich, St.louis, MO) to arrest cells in mitosis, then cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), and 1% non-essential amino acids (all from Invitrogen, Paisley, UK). The hESCs were cultured in DMEM-F12 with 20% Knockout Serum Replacement (KSR) in 1 mM L-glutamine, 10 mM nonessential amino acids (Invitrogen), 50 mM β-mercaptoethanol (Sigma), and 4 ng/ml FGF-2 (R&D Systems, Minneapolis, MN). For differentiation passaged cells were seeded on 5% Matrigel-Growth Factor Reduced (R&D Systems) and left for 48 h in maintenance medium. Growth media was then changed to DMEM/F12 containing 1 mM nonessential amino acids, L-glutamine, 100 ng/ml FGF-2, Activin A and/or BMP-4 (R&D systems) and left for four days. The concentration of serum (FBS) was 0% for the first day, 0.2% for the second day and 2% for third and fourth day. Cells were then grown for eight days in DMEM/F12 containing 10%FBS, 1 mM nonessential amino acids, L-glutamine, 1% dimethyl sulfoxide (DMSO) and 100 ng/ml HGF (R&D systems). Finally cells were grown for four days in DMEM/F12 containing 10%FBS, 1 mM nonessential amino acids, L-glutamine, 10^{-7} dexamethasone and/or 100 ng/ml oncostain M (Sigma-Alrich).

2.2. Western blotting

Cells were lysed in modified RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% sodium deoxycholate) containing protease (Roche, Mannheim, Germany) and phosphatase (Sigma) inhibitors. Protein concentrations were determined using BCA (Pierce Biotechnology, Rockford, IL). Antibodies used were goat anti-FoxA2, goat anti-GATA4 or goat anti-Sox17 from (R&D Systems) rabbit anti-E-cadherin, rabbit anti-brachyury, rabbit anti-IGF-1R^{Y1135} rabbit anti-IGF-1R, rabbit anti-IRS1, rabbit anti-AKT, or rabbit anti-Erk1/2from (Cell Signaling Technology, Beverly, MA) mouse anti-Oct 3/4, rabbit anti-GAPDH or goat anti-HNF4α from (Santa Cruz Biotechnology, Santa Cruz, CA) rabbit anti-Sox7 or rabbit anti-AFP from (Sigma Aldrich) or rabbit-anti Nanog from (Abcam, Cambridge, UK). Secondary anti-rabbit/mouse/goat IgG horseradish peroxidase-conjugated antibodies were from (Amersham) followed by signal detection using enhanced luminescence Hyperfilm-ECL (Amersham).

2.3. Flowcytometry

Cells were fixed with 70% ethanol, incubated with anti-ASGPR1 antibody (Abcam) for 1 h at room temperature (RT), washed with PBS (Hyclone, Logan, UT) containing 1% bovine serum albumin (BSA) and incubated with goat anti-rabbit Alexa Fluor 488-conjugated antibody (Invitrogen). Following analysis with flow cytometry, the data were processed with the Cell Quest software (Becton Dickinson, San Jose, CA).

2.4. ELISA albumin assay

Culture medium was replaced 3 days prior to the assay with 1.5 ml blank DMEM medium. Conditioned medium was harvested and assayed for albumin secretion using Human Albumin ELISA Quantitation kit (Bethyl Laboratory, Inc., Montgomery, TX) following the manufacturer's instructions. Albumin secretion levels were normalized with total protein of differentiated hepatocyte like cells at concentration of 0.125 mg/ml protein.

2.5. Urea assay

Culture medium was replaced 3 days prior to the assay with 1.5 ml blank DMEM/F12 medium. Conditioned medium was harvested and assayed for albumin secretion using urea enzymatic assay kit (Bioo Scientific, Austin, TX) following the manufacturer's instructions. Urea secretion levels were normalized with total protein of differentiated hepatocyte like cells at concentration of 0.27 mg/ml protein.

2.6. Periodic acid schiff (PAS) staining

The PAS staining system was purchased from Sigma-Aldrich. The differentiated cells were fixed in 4% paraformaldehyde for 20 min and then intracellular glycogen was stained using PAS staining according to the manufacturer's instructions.

3. Results

Optimization of a protocol for efficient differentiation of hepatocyte-like cells from human embryonic stem cells.

We optimized a three-step protocol for human hepatocyte differentiation based on the understanding of the mechanisms underlying mouse embryogenesis and modifications of previous reported protocols [11,12]. Fig. 1A illustrates the three differentiation steps (endoderm, hepatocyte specification and hepatocyte maturation) and the procedures we used to reach each of them. Human embryonic stem cells (hESCs) were maintained on mitotically inactive mouse embryonic fibroblast (MEF) feeder layer in embryonic stem cell (ES) culture media. Under these conditions hESCs expressed pluripotency markers, including Oct3/4 and Nanog (Fig. 1B). To initiate differentiation, hESCs were cultured on Matrigel-Growth Factor Reduced® (see Material and methods) for two days, followed by culturing in DMEM/F12 media containing FGF-2, gradual concentration of serum and various concentrations of Activin A and (bone morphogenetic protein 4) BMP4 (0, 20, 40, 60 and 80 ng/ml) (Fig. 1B). After 4 days of culture, the simultaneous addition of BMP4 resulted in higher expression of definitive endoderm markers as compared with Activin A treatment alone; GATA binding protein 4 (GATA4), sex determining region Y box 17 (SOX17), foreheads box A2 (FOXA2), while expressing lower levels of Oct3/4 and Nanog (Fig. 1B). Surprisingly, BMP4 concentrations higher than 40 ng/ml induced differentiation of extra-embryonic endoderm as indicated by the increased expression of SOX7 (Fig. 1B). Based on these data, we chose to use 60 ng/ml Activin A and 40 ng/ml BMP4 in the subsequent differentiation experiments. Culturing cells in RPMI supplied with B27 as described in other studies [11] resulted in lower degree of endoderm differentiation, and RPMI alone in lower survival rate (data not shown).

Cultures of induced definitive endodermal cells were next moved to DMEM/F12 supplemented with 10% serum, 1% DMSO and 100 ng/ml hepatocyte growth factor (HGF) and maintained for 7 days. As shown in Fig. 1C, cell cultures supplemented with 10% serum and DMSO/HGF resulted in diminished Oct3/4, Nanog, SOX7 and FoxA2 expression but initiated expression of AFP and hepatocyte nuclear factor 4α (HNF4α). For induction of the final stage of differentiation (mature hepatocytes) we cultured the specified hepatocytes in DMEM/F12 supplemented with 10% serum and either dexamethasone or oncostatin M (OSM) or a combination of both. In contrast to the earlier stages, expression of albumin appeared in the mature hepatocytes (Fig. 1C). After 5 days of culture, cells treated with the combination of both DEX and OSM were found to express the highest levels of the hepatocyte marker albumin as assayed by western blotting (Fig. 2A). Based on flow cytometry analyses the mature hepatocytes exhibited similar expression of the hepatocyte marker Asialoglycoprotein receptor 1 (ASGPR1) when treated with DEX alone or DEX + OSM (66 and 68.6%, respectively), while it was somewhat lower with OSM alone (45.6%) (Fig. 2B). Culturing of the hepatocyte-like cells for an

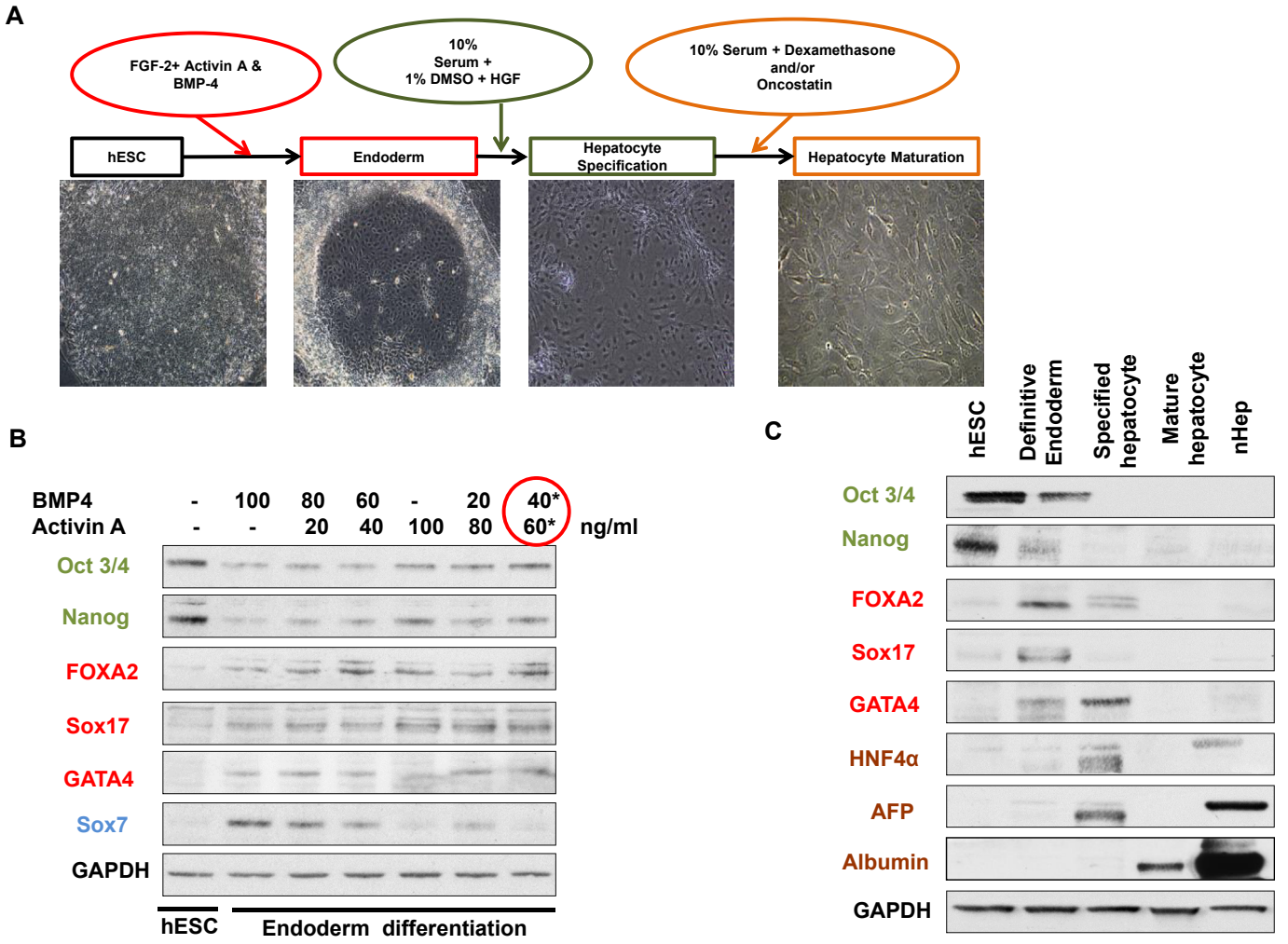

Fig. 1. Generation of hepatocyte-like cells from hESC. (A) Schematic presentation of the used differentiation protocol. See text. (B) WA01 cells (a hESC cell line) grown under feeder free conditions on reduced-growth factor Matrigel treated with FGF-2 (100 ng/ml) and indicated concentrations of BMP4 and Activin A for 4 days. Cells were then harvested, lysed for western blot analysis and blotted against stemness protein markers (Oct3/4, Nanog) (green), definitive endoderm markers (FOXA2, Sox17 and GATA4) (red) and extra-embryonic endoderm markers (Sox7) (blue). GAPDH was used as loading control. (C) Expression profiles of protein markers and transcription factors in the different stages of hepatocyte differentiation assayed by western blot analysis; stemness protein markers (green), definitive endoderm markers (red) and hepatic markers (HNF4a, AFP and Albumin) (brown). nHep was used as positive control. Data are representative of three or more independent experiments. FGF-2: Fibroblast growth factor-2; BMP4: Bone morphogenetic protein 4; Dex: Dexamethasone; OSM: Oncostatin M; hESC: human embryonic stem cells, nHep: Adult normal hepatocyte. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

additional week in complete hepatocyte basal media (HBM) significantly increased the percentage of ASGPR1 positive cells to 86.5% and also increased the level of albumin expression (data not shown).

At the completion of the differentiation protocol, the hepatocyte-like cells were shown to display several known hepatic functions. Periodic acid-Schiff (PAS) staining indicated glycogen synthesis by the differentiated cells, in addition to urea and albumin secretion (Fig. 2C–E). Moreover, the morphology of the differentiated cells also shared many characteristics with primary hepatocytes, including a large cytoplasmic-to-nuclear ratio, prominent nucleoli and several cells were found to be binucleated (Fig. 2C). Regarding glycogen synthesis and urea production no significant difference was found between OSM, Dex or Dex/OSM treated cells. However, the combination of Dex/OSM treatment resulted in highest albumin secretion, i.e. two-fold of that by OSM alone, and four-fold of that by Dex alone (Fig. 2D). Again, culturing of cells for an additional week in complete HBM media significantly increased the albumin and Urea secretion (data not shown). Based on these data, we chose to use the combination of Dex/OSM in the following experiments.

3.1. Downregulation of IGF-1R expression during hepatocyte differentiation stages

After establishing the differentiation protocol, we studied the expression of IGF-1R during hepatocyte differentiation of hESC by western blotting. During endoderm differentiation IGF-1R expression was slightly reduced by (8%). However, a dramatic and statistical significant reduction (more than 50%) was shown during both the hepatocyte specification and maturation stages (Fig. 3A and B). As expected, the nHep cells expressed hardly any IGF-1R. Next we investigated the expression of IGF-1R downstream signaling proteins; i.e. insulin receptor substrate-1 (IRS1), ERK (MAPK pathway) and AKT (PI3K/AKT pathway). As shown, AKT was clearly increased in all 3 differentiation stages compared to hESC (Fig. 3C). IRS1 was slightly increased in the hepatocyte specification and maturation steps, while ERK1/2 appeared only in these two stages and was

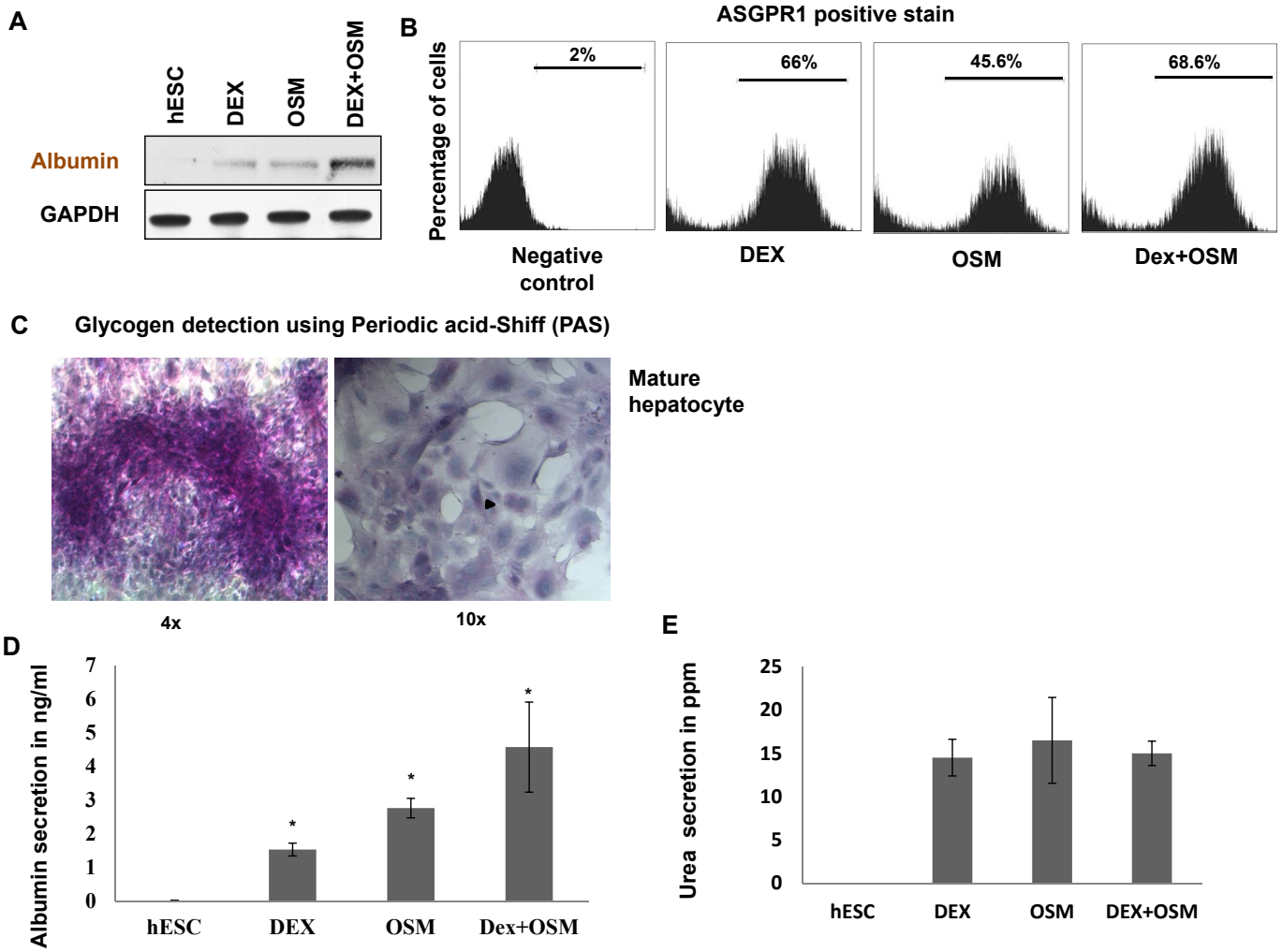

Fig. 2. Characteristics of generated hepatocyte-like cells. (A) Western blot analysis showing expression of hepatocyte marker albumin in mature hepatocytes generated by DEX and/or OSM. hESC was used as negative control. (B) Flowcytometric analysis showing percentage of mature hepatocytes, generated by DEX and/or OSM, positive for hepatocyte surface marker ASGPR1. The negative control was obtained by omitting the primary antibody. (C) Periodic and Schiff staining of mature hepatocytes generated by DEX and OSM. Magnification 4× and 10×. Black arrow indicates binucleated cells. (D) Secreted albumin levels in the culture media of mature hepatocytes generated by DEX and/or OSM. The concentration of albumin in (ng/ml) was assessed by ELISA. Albumin secretion levels were normalized with total protein of differentiated hepatocyte-like cells at a concentration of 0.125 mg/ml protein. Data are presented as means ± SDs of three independent experiments and considered significant (*) at $P < 0.01$ using one way ANOVA. (E) Secreted urea levels in the culture media (ppm) of mature hepatocytes, generated by DEX and/or OSM, were assessed by urea assay kit. Secretion levels were normalized with total protein of differentiated hepatocyte-like cells at a concentration of 0.27 mg/ml protein. Data are presented as means ± SDs of three independent experiments. ELISA: enzyme linked immunosorbent assay, ppm: parts per million.

strongest in mature hepatocytes. Thus, the downstream signaling proteins were expressed in a manner opposite to the IGF-1R. All of them, although at a much lower level, were also expressed in nHep (Fig. 3C).

3.2. Minor role of IGF-1R in endodermal differentiation

To investigate the potential role of IGF-1R in endodermal differentiation, NVP-AEW 54, a selective inhibitor of IGF-1R phosphorylation, was used to block the IGF-1R activity and IGF-1 ligand was used to increase activity of IGF-1R during the endoderm differentiation. Neither inhibition nor activation of IGF-1R showed any clear effect on endoderm differentiation as indicated by unchanged expression levels of the endoderm markers GATA4 and SOX17 (Fig. 4). However, IGF-1R inhibition resulted in a slight reduction in Nanog and Oct3/4 expression as compared to the control (Fig. 4), and IGF-1 stimulation showed a slight increase in extra-embryonic endoderm (SOX7) and mesoderm (as assayed by brachyury expression) induction, with no effect on ectoderm induction (E-cadherin) (Fig. 4).

4. Discussion

Normal hepatocytes (nHeps) do not express any detectable levels of IGF-1R mRNA or protein [9,10], whereas hESC have been shown to express high levels of IGF-1R [8]. This suggests a possible role for IGF-1R in hepatocyte differentiation. In the current study we aimed to investigate the expression pattern of IGF-1R and the downstream signaling pathway during hepatocyte differentiation process. To do so, we modified a step-wise differentiation protocol from hESC based on previous studies [11,12]. Direct differentiation of hepatocytes from hESC has been previously reported as well [13–15]. However, more and more studies have suggested that the step-wise differentiation protocol, in a pattern similar to the embryological development, would be more efficient for hepatocyte differentiation [11,12].

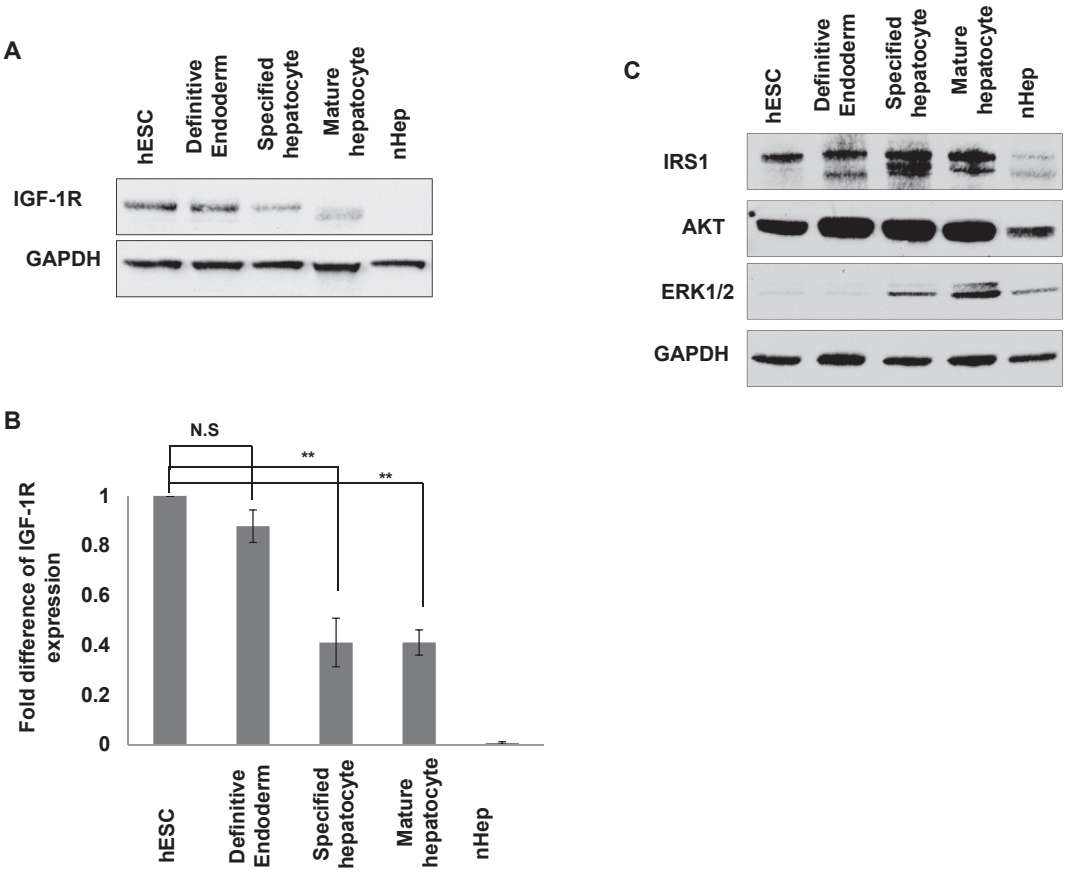

Fig. 3. Downregulation of IGF-1R expression during hepatocyte differentiation. (A–C) Cells in the different stages of hepatocyte differentiation (obtained as described in Fig. 1 and the text) were compared for IGF-1R, IRS1, AKT and ERK 1/2 expression using western blot. nHep was used as negative control. GAPDH was loading control. (B) Densitometric analysis of the Western blots in (A) normalized to GAPDH expression. Data are presented as fold-difference compared to nHep. Data are presented as means ± SDs of three independent experiments and considered significant (**) at $P < 0.05$ using two-sided *t*-test. N.S: non-significant. IGF-1R: insulin like growth factor −1 receptor.

It is well known that endodermal formation *In vivo* requires nodal signaling [16,17], and treatment of hESCs with Activin A have been shown to initiate expression of endodermal transcriptional regulators and markers similar to embryological development [11,12,18]. As combination of BMP4 and Activin A has been reported to be more efficient to promote definitive endodermal formation [11,19], we investigated various concentrations of Activin A and BMP4. We found that Activin A and BMP4 at doses of 60 ng/ml and 40 ng/ml, respectively, were optimal for definitive endoderm differentiation. Higher BMP4 concentrations induced formation of extraembryonic endoderm.

Stepwise additions of FGF, HGF, dexamethasone and/or OSM drive initiation of similar transcriptional events as in liver organogenesis [11,12] mimicking the secretion of signaling molecules from the underlying mesoderm [20]. We found that addition of FGF-2 and HGF following BMP4 and FGF-2 during endoderm induction mediated the continuation of FOXA2 and GATA4 expression at lower levels. The expression of GATA4 and FOXA2 has been shown to be important for early hepatic gene expression [21,22] and act as mediators for competence in foregut endoderm [23]. The addition of FGF-2 and HGF also initiated expression of HNF4α which acts as an essential regulator for hepatocyte differentiation [24]. For hepatocyte maturation, we found that the combination of both OSM and dexamethasone was the most efficient in inducing hepatic functions, at least regarding albumin production and secretion. At the end of the differentiation protocol 70% of generated hepatocyte-like cells were expressing the hepatocyte marker (ASGPR1), higher than the reported percentage of cells from previous studies [11,12]. In addition, they were able to secrete urea and albumin and to synthesis and store glycogen indicating the functional ability of the cells. The lower level of albumin secretion from the generated hepatocyte-like cells in our study compared to the previous ones [11,12] could be explained by the lower number of cells used for albumin detection, which was equivalent to 125 μg/ml protein. The fact that, nHep cells were expressing higher levels of albumin than the differentiated hepctocyte-like cells, was also reported previously as limitation in the differentiation protocol [11,12]. However, we also found the nHep in our study to express detectable levels of AFP, which might indicate fetal origin of the donor nHep [25].

After optimizing the hepatocyte differentiation protocol, we investigated the expression pattern of IGF-1R and the downstream signaling proteins. With the exception of the slight elevated expression level of AKT, we did not detect any significant changes in expression levels of IGF-1R, ERK 1/2 or IRS1 during endoderm differentiation. However, after the hepatic linage commitment starting from hepatocyte specification stage a significant reduction in IGF-1R expression occurred. This was paralleled with continued high level of AKT and increased expression of IRS1 and ERK1/2. Together, this suggests that the shift to IGF-1R downregulation and simultaneous upregulation of downstream signaling, starting off upon hepatic linage commitment, may have a regulatory role in hepatocyte differentiation.

The importance of ERK/MAPK pathway and the PI3K/AKT pathway in hepatocyte differentiation have been pointed out in a number of previous studies. Magner et al., 2013 showed that

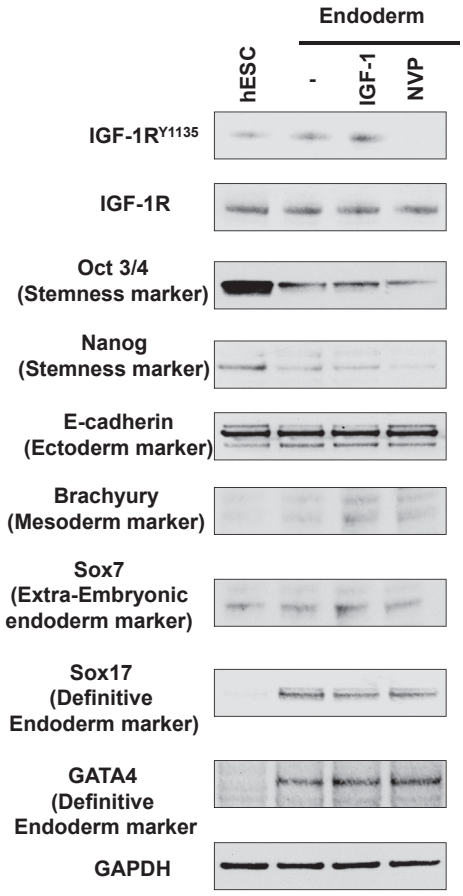

Fig. 4. IGF-1R has a minor role in endoderm differentiation. hESC were seeded on reduced-growth factor Matrigel for 48 h, then treated with either 1 μM NVP-AEW541, 100 ng/ml IGF-1 or vehicle for four days and proceeded to definitive endoderm as described in the differentiation protocol (cf. Fig. 1A), followed by western blot analysis for the indicated markers. Activation and inhibition of IGF-1R was indicated by levels of IGF-IR^{Y1135} phosphorylation. GAPDH (black) was used as loading control. Data are representative of three or more independent experiments.

inhibition of the PI3K/AKT pathway after endoderm differentiation inhibited hepatocyte differentiation [26], and Calmont et al., 2006 showed FGF mediated induction of hepatic genes function through the MAPK pathway [27]. In addition other studies have demonstrated the importance of both PI3K/AKT and ERK/MAPK pathway in hepatocyte regeneration after partial hepatectomy [28–30]. These latter studies provide further explanation for the lower expression of AKT and ERK 1/2 in adult normal hepatocytes as normal hepatocytes were reported to restore lower levels of AKT and ERK after liver regeneration [28–30]. Similarly IRS1 was also reported to restore lower levels after liver regeneration [31].

Further confirmation for the possible regulatory role of IGF-1R after hepatic linage commitment came from the observation that neither inhibition nor activation of IGF-1R had any significant effect on endoderm differentiation markers. Although it is important to point out that inhibition of IGF-1R promoted more efficient differentiation and IGF-1R stimulation enhanced the mesoderm, extraembryonic endoderm differentiation. It's also important to note that our results are not in line with some of the findings from the previous study by Magner et al., 2013, which showed that IGF-1R expression levels remain the same during hepatocyte differentiation. As the previous mentioned study was the only one to report detectable levels of IGF-1R in differentiated hepctocyte [9,10], this suggests more investigations about the role of IGF-1R in hepctocyte differentiation are needed.

In conclusion, our data suggests that changes in expression of IGF-1R and downstream signaling proteins occurring after initiation of hepatic linage commitment may play a regulatory role for hepatocyte differentiation from embryonic stem cells.

Acknowledgements

This work was supported by the Swedish Cancer Foundation (#CAN215/43), the Swedish Research Council (#D0356401), the Cancer Society in Stockholm (#141232), the Swedish Children Cancer Society (#PROJ12/020), the Stockholm County Council, and Karolinska Institutet.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.08.157.

Conflict of interest

The authors declare no conflict of interest.

References

[1] R. Baserga, M. Resnicoff, M. Dews, The IGF-I receptor and cancer, Endocrine 7 (1997) 99–102.
[2] D. LeRoith, C.T. Roberts Jr., The insulin-like growth factor system and cancer, Cancer Lett. 195 (2003) 127–137.
[3] M.N. Pollak, Insulin-like growth factors and neoplasia, Novartis Found. Symp. 262 (2004) 84–98 discussion 98-107, 265-108.
[4] B. Valentinis, R. Baserga, IGF-I receptor signalling in transformation and differentiation, Mol. Pathol. MP 54 (2001) 133–137.
[5] J. Nakae, Y. Kido, D. Accili, Distinct and overlapping functions of insulin and IGF-I receptors, Endocr. Rev. 22 (2001) 818–835.
[6] B. Valentinis, A. Morrione, F. Peruzzi, et al., Anti-apoptotic signaling of the IGF-I receptor in fibroblasts following loss of matrix adhesion, Oncogene 18 (1999) 1827–1836.
[7] M. Ayatollahi, M. Soleimani, B. Geramizadeh, et al., Insulin-like growth factor 1 (IGF-I) improves hepatic differentiation of human bone marrow-derived mesenchymal stem cells, Cell Biol. Int. 35 (2011) 1169–1176.
[8] J.M. Sperger, X. Chen, J.S. Draper, et al., Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 13350–13355.
[9] K. Breuhahn, P. Schirmacher, Reactivation of the insulin-like growth factor-II signaling pathway in human hepatocellular carcinoma, World J. Gastroenterology WJG 14 (2008) 1690–1698.
[10] J.G. Scharf, T. Braulke, The role of the IGF axis in hepatocarcinogenesis, Hormone Metabolic Res. – Hormon- und Stoffwechselforschung – Hormones metabolisme 35 (2003) 685–693.
[11] K. Si-Tayeb, F.K. Noto, M. Nagaoka, et al., Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells, Hepatology 51 (2010) 297–305.
[12] H. Basma, A. Soto-Gutierrez, G.R. Yannam, et al., Differentiation and transplantation of human embryonic stem cell-derived hepatocytes, Gastroenterology 136 (2009) 990–999.
[13] R.E. Schwartz, J.L. Linehan, M.S. Painschab, et al., Defined conditions for development of functional hepatic cells from human embryonic stem cells, Stem Cells Dev. 14 (2005) 643–655.
[14] D.C. Hay, D. Zhao, J. Fletcher, et al., Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo, Stem Cells 26 (2008) 894–902.
[15] D.C. Hay, D. Zhao, A. Ross, et al., Direct differentiation of human embryonic stem cells to hepatocyte-like cells exhibiting functional activities, Cloning Stem Cells 9 (2007) 51–62.
[16] F.L. Conlon, K.M. Lyons, N. Takaesu, et al., A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse, Development 120 (1994) 1919–1928.
[17] M.Y. Wu, C.S. Hill, Tgf-beta superfamily signaling in embryonic development and homeostasis, Dev. Cell 16 (2009) 329–343.
[18] K.A. D'Amour, A.D. Agulnick, S. Eliazer, et al., Efficient differentiation of human embryonic stem cells to definitive endoderm, Nat. Biotechnol. 23 (2005) 1534–1541.
[19] A.K. Teo, Y. Ali, K.Y. Wong, et al., Activin and BMP4 synergistically promote formation of definitive endoderm in human embryonic stem cells, Stem Cells 30 (2012) 631–642.
[20] K.S. Zaret, Regulatory phases of early liver development: paradigms of organogenesis. Nature reviews, Genetics 3 (2002) 499–512.

[21] J.D. Molkentin, Q. Lin, S.A. Duncan, et al., Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis, Genes & Dev. 11 (1997) 1061–1072.
[22] C.T. Kuo, E.E. Morrisey, R. Anandappa, et al., GATA4 transcription factor is required for ventral morphogenesis and heart tube formation, Genes & Dev. 11 (1997) 1048–1060.
[23] K.H. Kaestner, H. Hiemisch, G. Schutz, Targeted disruption of the gene encoding hepatocyte nuclear factor 3gamma results in reduced transcription of hepatocyte-specific genes, Mol. Cell. Biol. 18 (1998) 4245–4251.
[24] J. Li, G. Ning, S.A. Duncan, Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha, Genes & Dev. 14 (2000) 464–474.
[25] E. Ruoslahti, M. Seppala, Studies of carcino-fetal proteins: physical and chemical properties of human alpha-fetoprotein, Int. J. Cancer. J. Int. du Cancer 7 (1971) 218–225.
[26] N.L. Magner, Y. Jung, J. Wu, et al., Insulin and IGFs enhance hepatocyte differentiation from human embryonic stem cells via the PI3K/AKT pathway, Stem Cells 31 (2013) 2095–2103.
[27] A. Calmont, E. Wandzioch, K.D. Tremblay, et al., An FGF response pathway that mediates hepatic gene induction in embryonic endoderm cells, Dev. Cell 11 (2006) 339–348.
[28] T.G. Araujo, A.G. de Oliveira, N. Tobar, et al., Liver regeneration following partial hepatectomy is improved by enhancing the HGF/Met axis and Akt and Erk pathways after low-power laser irradiation in rats, Lasers Med. Sci. 28 (2013) 1511–1517.
[29] H. Talarmin, C. Rescan, S. Cariou, et al., The mitogen-activated protein kinase kinase/extracellular signal-regulated kinase cascade activation is a key signalling pathway involved in the regulation of G(1) phase progression in proliferating hepatocytes, Mol. Cell. Biol. 19 (1999) 6003–6011.
[30] M. Li, X. Zhou, J. Mei, et al., Study on the activity of the signaling pathways regulating hepatocytes from G0 phase into G1 phase during rat liver regeneration, Cell. Mol. Biol. Lett. 19 (2014) 181–200.
[31] C. Desbois-Mouthon, D. Wendum, A. Cadoret, et al., Hepatocyte proliferation during liver regeneration is impaired in mice with liver-specific IGF-1R knockout, FASEB J. Official Publ. Fed. Am. Soc. Exp. Biol. 20 (2006) 773–775.