

Chromatin remodelling, a novel strategy to expedite the hepatic differentiation of adult bone marrow stem cells *in vitro*

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Abstract

Background: Covalent modification of histones is key in processes that determine lineage-specific gene expression and cell fate decisions. In addition, histone deacetylase inhibition favours the maintenance of the *in vivo* hepatocellular phenotype of primary hepatocyte cultures. Here, it was investigated whether the histone deacetylase inhibitor (HDACi) Trichostatin A (TSA) could promote the hepatic differentiation potential of rat mesenchymal progenitor cells (rMPC) derived from adult bone marrow. 1 μ M TSA was added to rMPC that were exposed to hepatogenic factors, either as a mixture (cf. cocktail-TSA condition) or sequentially according to their secretion during the liver embryogenesis *in vivo* (cf. sequential-TSA condition).

Results: Exposure of the cells to TSA considerably improved endodermal differentiation regardless of the set-up used. More specifically, TSA sped up the appearance of binucleated polygonal-shaped cells, and promoted chronological expression of hepatic proteins, including hepatocyte nuclear factor (HNF)3 β , alpha-fetoprotein (AFP), albumin (ALB), cytokeratin (CK)18 and HNF1 α . Hepatic functionality could only be improved in cultures using the sequential-TSA condition, as evidenced by significantly upregulated ALB-secretion to levels found in primary hepatocyte cultures.

Conclusions: The strategy using HDACi to significantly promote, in time and in number, the *in vitro* differentiation of rMPC into hepatocytes, is novel and opens new perspectives.

Keywords: bone marrow, adult stem cells, hepatocytes, epigenetic modification, liver embryonic development

Introduction

Functional hepatic-based *in vitro* models are suitable in drug development, in particular to gain an early stage information with respect to the efficacy and safety of new chemical entities (Ragan, 2006). Several models are available; yet, they spontaneously dedifferentiate in culture (Papeleu, 2002; Elaut, 2006). Current strategies of our laboratory are centred at (re)-establishing and maintaining the expression of important liver-regulating transcription factors via direct interference with the local chromatin structure (Papeleu, 2003; Vanhaecke, 2004a; Vinken, 2006; Snykers, 2007a; Henkens, 2007). Epigenetic modification, including acetylation and deacetylation of histones, contributes to tissue-specific gene expression (Kuo, 1998). Whereas actively transcribed genes are characterised by highly acetylated core histones, hypoacetylated histones are preferentially found in transcriptionally silenced chromatin regions (Grunstein, 1997). Inhibition of HDAC leading to

histone hyperacetylation is therefore associated with gene transcription.

Our group previously showed that exposure of primary hepatocyte cultures to TSA, the reference compound of hydroxamate-based HDACi, induces cell cycle arrest, prolongs lifespan and improves their differentiated state (Papeleu, 2003; Vanhaecke, 2004a; Vinken, 2006; Henkens, 2007). Consequently, the hypothesis was made that epigenetic alterations could represent a novel approach to develop phenotypically stable primary hepatocyte cultures. Since chromatin remodelling also plays a central role during organogenesis in regulating differentiation and stem cell functions (Cerny, 2004; Snykers, 2007a, Kiefer, 2007; Liu, 2007), we investigated here whether TSA-exposure could also expedite and improve hepatic differentiation of adult bone marrow stem cells. Indeed, in recent years postnatal progenitors became considered as an alternative and tentatively unlimited source of endodermal cells, including hepatocytes

(Kang 2005; Lange, 2005; Ong, 2006; Aurich, 2006). Our group recently succeeded to produce functional hepatocytes out of rMPC derived from adult bone marrow via serial incubation with hepatogenic factors, mimicking their secretion pattern during liver embryogenesis *in vivo* (Snykers, 2006). Here, we report the effects of 1 μ M TSA on the endodermal differentiation of rMPC under two different conditions, namely (i) the simultaneous exposure to a cocktail of hepatogenic factors and (ii) the treatment with the same hepatogenic factors but sequentially, reflecting the hepatogenesis *in vivo*.

Materials and methods

Isolation and culture of undifferentiated rMPC

rMPC were isolated from male Fisher rats (4-6 weeks old) and expanded as described by Jiang., 2002. Cell karyotyping was determined as previously described (Jiang., 2002). Rats had access to food

and water *ad libitum* and were housed according to the Institutional Animal Care and Use Committee guidelines from the University of Minnesota.

Hepatocyte differentiation of rMPC

Hepatic differentiation was performed as previously described (Snykers, 2007b).

Immunocytochemistry

Immunostaining was carried out as previously described (Snykers, 2006).

Microscopic analysis

Cell morphology was analysed using phase-contrast light-microscopy (Nikon).

Albumin ELISA

ALB levels, secreted into the culture media, were analysed by ELISA (Koebe, 1994).

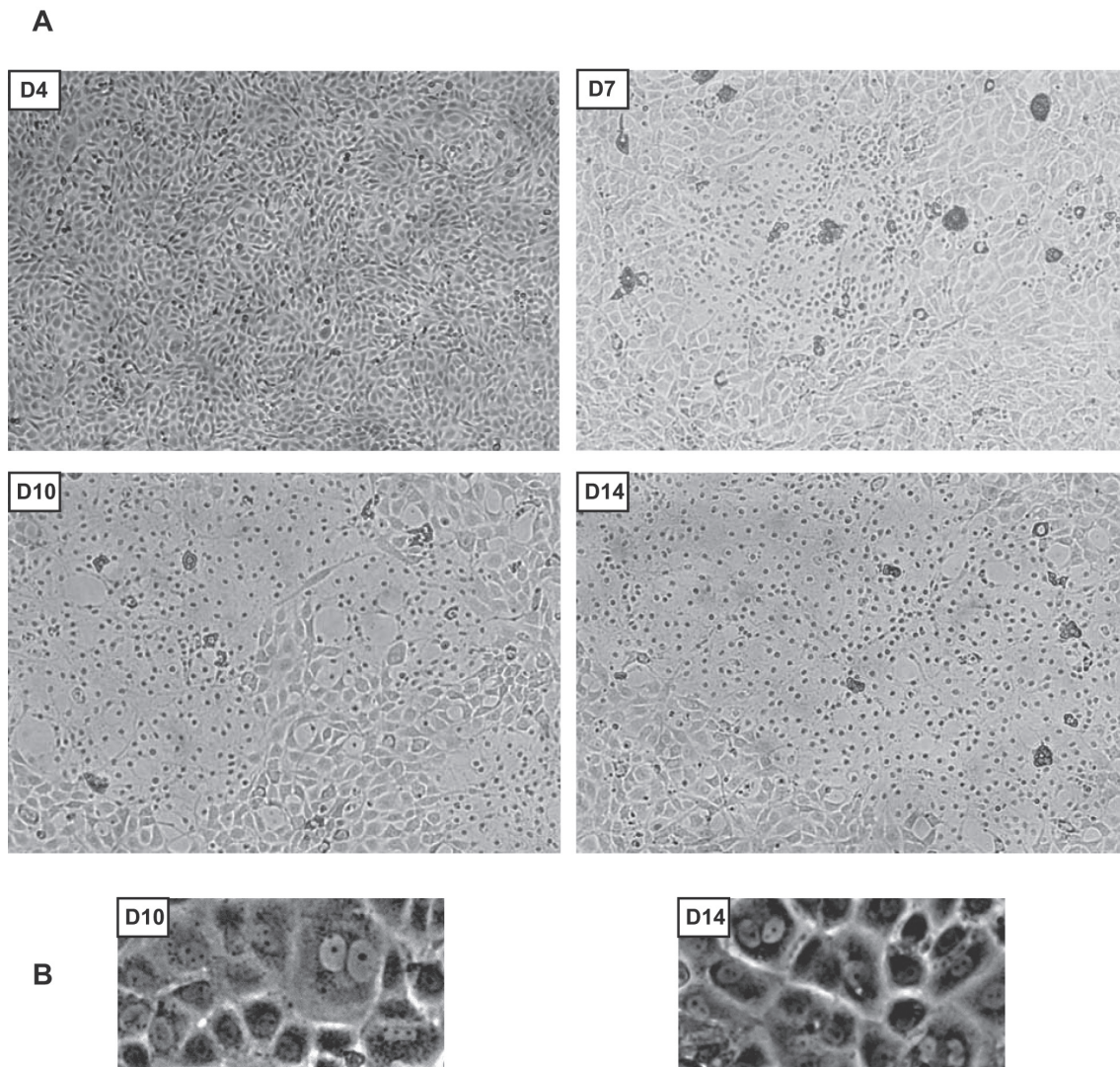


Fig. 1: Cell morphology. (A) Light-microscopic analysis of rMPC under sequential-TSA conditions (B). Pictures were magnified by 3-fold for better observation of binucleated cells; 10x10 and 30x10 original magnification, phase contrast. Similar pictures were obtained for rMPC under cocktail-TSA conditions.

Table 1: Characterization of hepatic differentiation of rMPC under sequential (+/- TSA) and cocktail (+/- TSA) conditions at the protein level. Immunocytochemistry was performed for HNF3 β , AFP, ALB, CK18, and HNF1 α . Values indicate the percentage of positive immunostained cells and represent means of at least 4 independent experiments. S (sequential), ST (sequential-TSA), C (cocktail) and CT (cocktail-TSA).

Day of culture	HNF3 β				AFP			
	S	ST	C	CT	S	ST	C	CT
D6	8 \pm 2	58 \pm 1	0 \pm 0	42 \pm 10	77 \pm 14	81 \pm 25	25 \pm 7	79 \pm 26
D10	40 \pm 13	71 \pm 11	2 \pm 1	12 \pm 5	10 \pm 1	75 \pm 17	3 \pm 0	76 \pm 10
D12	92 \pm 8	93 \pm 3	11 \pm 5	73 \pm 10	0 \pm 0	77 \pm 12	0 \pm 0	78 \pm 8
D16	54 \pm 20	71 \pm 11	24 \pm 7	57 \pm 12	0 \pm 0	NT	0 \pm 0	NT
D18	0 \pm 0	8 \pm 4	0 \pm 0	10 \pm 5	0 \pm 0	79 \pm 17	0 \pm 0	83 \pm 16

Day of culture	ALB				CK18				HNF1 α			
	S	ST	C	CT	S	ST	C	CT	S	ST	C	CT
D6	0 \pm 0	42 \pm 20	0 \pm 0	9 \pm 6	0 \pm 0	16 \pm 1	0 \pm 0	2 \pm 1	0 \pm 0	13 \pm 5	0 \pm 0	7 \pm 5
D10	NT	75 \pm 5	0 \pm 0	9 \pm 6	0 \pm 0	76 \pm 16	0 \pm 0	28 \pm 7	0 \pm 0	48 \pm 1	0 \pm 0	33 \pm 3
D12	36 \pm 3	81 \pm 7	12 \pm 1	50 \pm 8	77 \pm 6	94 \pm 5	33 \pm 3	55 \pm 33	35 \pm 11	63 \pm 5	27 \pm 3	48 \pm 2
D16	81 \pm 3	85 \pm 6	24 \pm 4	66 \pm 7	90 \pm 7	92 \pm 11	38 \pm 1	86 \pm 6	64 \pm 10	78 \pm 10	21 \pm 8	64 \pm 4
D18	92 \pm 2	92 \pm 3	32 \pm 4	83 \pm 4	94 \pm 3	95 \pm 2	63 \pm 5	64 \pm 16	89 \pm 7	91 \pm 7	22 \pm 4	42 \pm 12

Statistics

Results are expressed as mean \pm sd. Statistical analyses were performed using Oneway Anova and Student's t-test. The significance level was set at 0.05.

Results

The effect of TSA on the hepatic differentiation process of rMPC

Morphological features

Upon TSA-exposure, colonies of small epithelial-to-polygonal-shaped cells appeared within the rMPC-culture, regardless of the set-up (Fig.1). As differentiation progressed, these cells increased in size and adopted a polygonal-to-cuboidal shape (Fig.1). Typical binucleated cells were present from day 10 onwards. In the absence of TSA, sequential cultures underwent similar morphologic changes, yet with a delay in time (Snykers, 2006). Under cocktail conditions, the population remained heterogeneous (Snykers, 2006).

Phenotypic changes during differentiation

We evaluated whether the morphological changes in TSA-exposed cells (cocktail-TSA and sequential-TSA cultures) versus non-exposed cells (cocktail and sequential cultures) are associated with increased changes in expression patterns at the protein level. Hereto, the expressions of immature (AFP, HNF3 β) and mature (ALB, CK18, HNF1 α) hepatic markers were analysed by immunofluorescence.

Both sequential-TSA and sequential cultures

progressively reached an adult expression pattern. The cells chronologically expressed HNF3 β , AFP, CK18, ALB and HNF1 α . Upon TSA-exposure, however, an earlier onset of expression and a higher number of positive stained cells were obtained (Fig.2, Table 1). Remarkably, TSA also supported hepatic maturation of the cells cultured under cocktail conditions, which would otherwise have remained immature (Fig.3, Table 1). The supportive effect of TSA was mostly pronounced in these cells. Nevertheless, sequential-TSA cultures still gained a higher degree of hepatic maturation (Table 1). More specifically, at days 16-18 about 86%, 94% and 85% of cells under sequential-TSA conditions expressed ALB, CK18 and HNF1 α , respectively whereas this number was limited to 75%, 75%, 53%, respectively, under cocktail-TSA conditions (Table 1). Besides provoking an inductive effect, TSA also prolonged the hepatic expression in both culture systems (Figs.2-3, Table 1). Complete maintenance of 'differentiation' could, however, not be achieved under the cocktail-TSA condition, as both mature HNF1 α and CK18 expressions declined at the end of culture time (Table1). On the other hand, expression of early AFP persisted throughout the whole differentiation process in both TSA-exposed conditions (Figs. 2-3, Table 1).

Hepatic functionality

To assess whether these hepatocyte-like cells derived from rMPC acquired typical functional hepatic features of primary hepatocytes, the potential

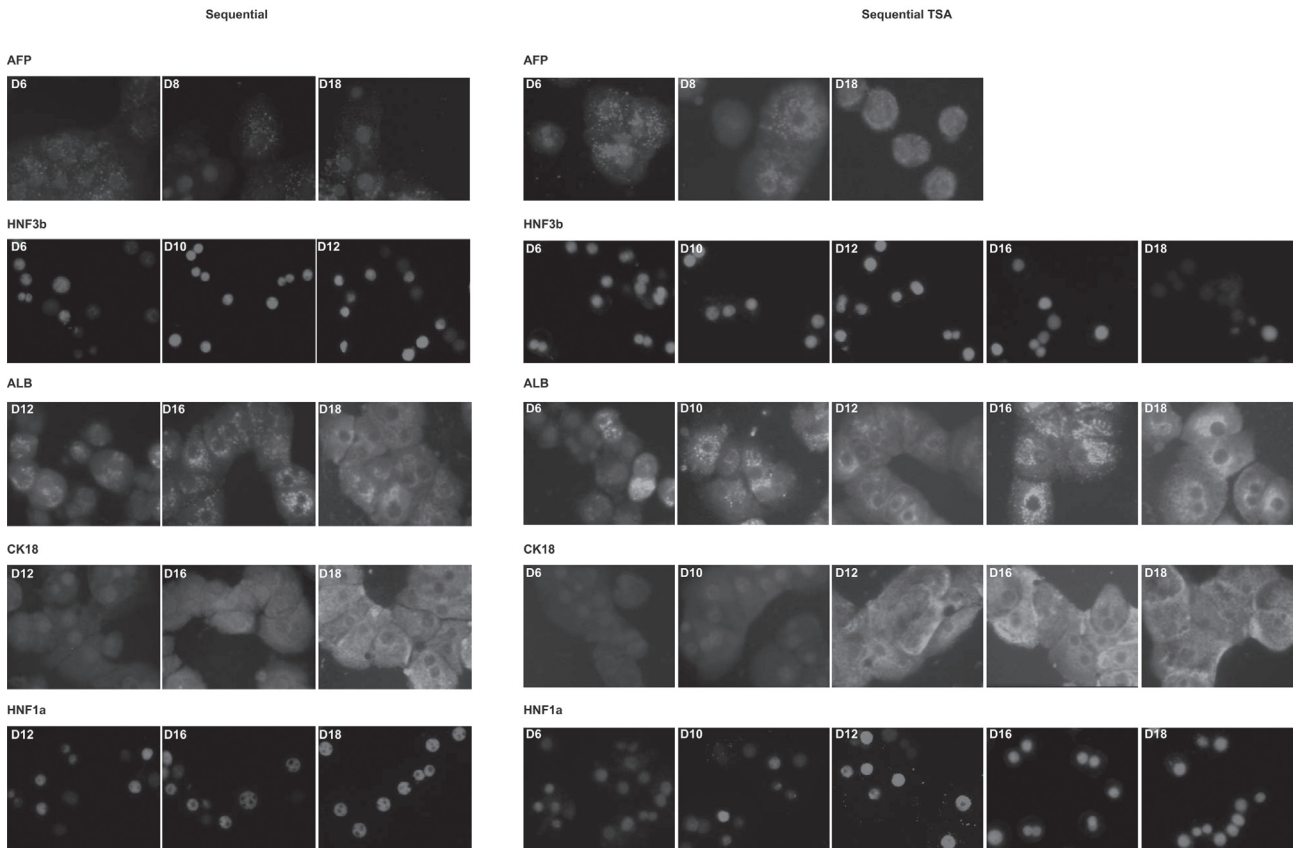


Fig. 2: Characterization of hepatic differentiation of rMPC under sequential +/- TSA conditions. Immunofluorescence was performed for HNF3 β -cy3, AFP-FITC, ALB-FITC, CK18-FITC, and HNF1 α -cy3. Pictures are shown from day 6 or onset of positive staining. 32x10 original magnification. Stainings have the same magnification and are representative for at least 4 separate experiments.

to secrete ALB was taken as a general parameter.

TSA, added to rMPC under sequential conditions significantly upregulated the ALB-secretion rate from day 15 onwards ($p < 0.01$, Oneway Anova and Student's t-test) when compared with regular sequential cultures (Fig.4). In contrast, only a minor, non-significant upregulation of the ALB-secretion could be detected upon application of TSA to cocktail cultures. More specifically, without co-addition of TSA, ALB-secretion just extended basal levels of 0.55 μ g/ml media.

Discussion

Research on the multipotent behaviour of postnatal bone marrow stem cells is progressively emerging. Both, successful and failed, hepatic differentiation have been reported. The type, concentrations and order of application of lineage-specific growth factors and cytokines used have been identified as critical factors in directing lineage-specific stem cell growth and differentiation (Moore, 2006). Recently, also chromatin modulation has gained interest as a strategy to modify cell fate. In fact, mediation of transcriptional regulation of gene expression not only occurs through specific interactions of transcription factors with the target DNA, but also through epigenetic mechanisms (Kuo, 1998; Vanhaecke,

2004b; Pitarque, 2005). These are reversible and involve structural chromatin modifications, e.g. histone (de)acetylation, and DNA methylation (Vanhaecke, 2004b). In general, although not exclusively, histone hyperacetylation is associated with gene transcription, whereas deacetylation of histones is associated with gene silencing (Grunstein, 1997; Kuo, 1998). HDAC inhibition is therefore linked with transcriptional activation.

Although mechanistic insights in transcriptional regulation and constitutive expression of liver-specific genes are at present largely unresolved, evidence for a predominant role of such epigenetic events in the acquisition and maintenance of the differentiated phenotype in cultured hepatocytes is accumulating. Indeed, we previously found that TSA-addition to isolated primary hepatocytes impedes cell cycling (G0-to-G1 cell cycle transition), significantly retards apoptosis and improves cell junction intercellular communication and xenobiotic biotransformation (Papeleu, 2003, 2005; Vanhaecke, 2004a; Vinken, 2006; Henkens, 2007). Also, covalent modification of histones are central in both processes that determine lineage-specific gene expression and cell fate decisions (Cerny, 2004; Snykers, 2007a; Torres-Padilla, 2007), and in the regulation of differentiation, normal development and stem cell functions during

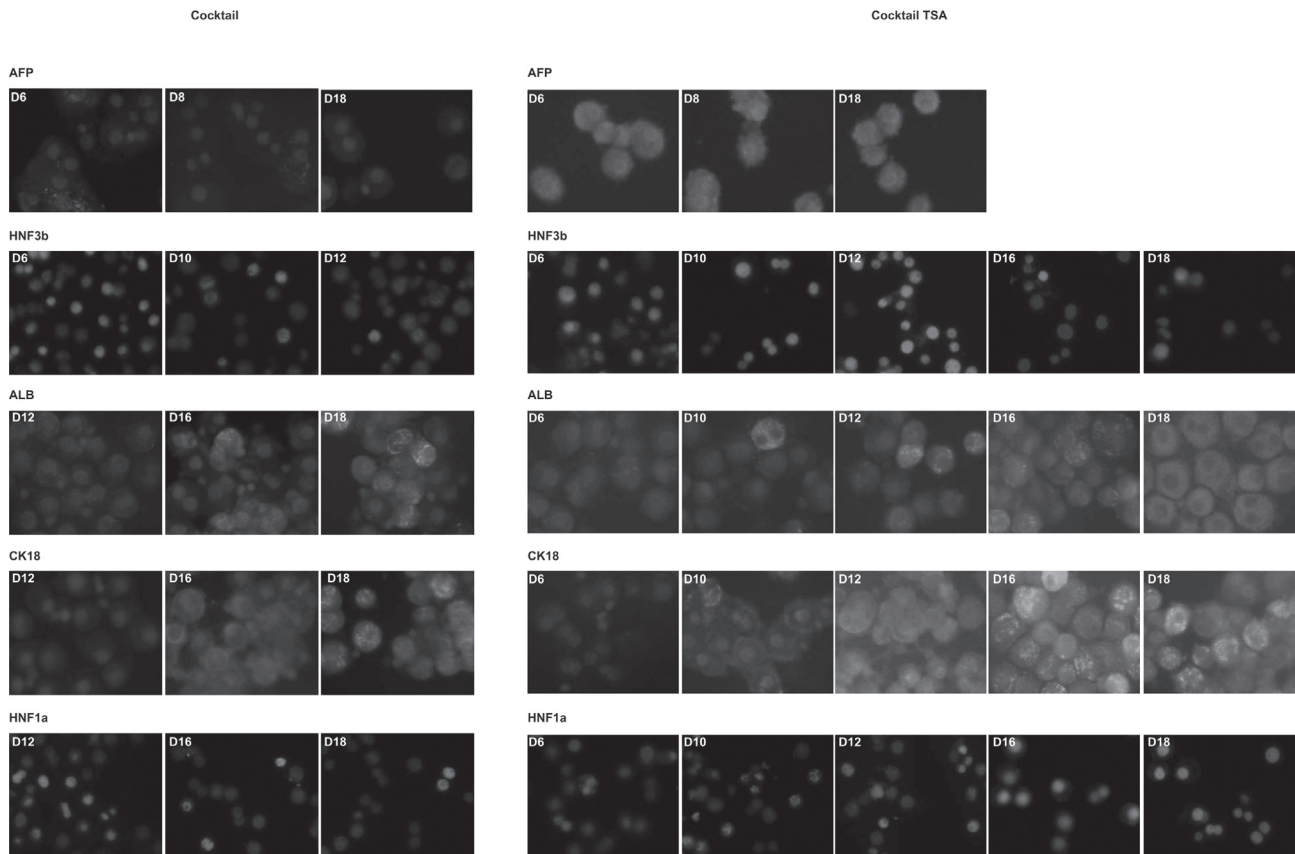


Fig. 3: Characterization of hepatic differentiation of rMPC under cocktail +/- TSA conditions. Immunofluorescence was performed for HNF3 β -cy3, AFP-FITC, ALB-FITC, CK18-FITC, and HNF1 α -cy3. Pictures are shown from day 6 or onset of positive staining. 32x10 original magnification. Stainings have the same magnification and are representative for at least 4 separate experiments.

embryonic organogenesis (Cerny, 2004; Snykers, 2007a; Kiefer, 2007; Liu, 2007). These findings encouraged us to investigate whether HDACi could also promote the hepatic potency of adult bone marrow-derived progenitors. Hereto, we evaluated the effect of TSA under two different culture conditions. (i) The 'cocktail condition' comprised simultaneous exposure to a mixture of well-defined hepatogenic factors (FGF4, HGF, ITS and dexamethasone). From previous work, this strategy was known to result in a heterogeneous population of hepatocyte-like cells with incomplete differentiation degree (Snykers, 2006). (ii) The more innovative 'sequential condition' involved sequential exposure of the cells to well-defined hepatogenic factors, reflecting liver embryogenesis *in vivo* (FGF4/HGF/HGF+ITS+dexamethasone). In contrast to the cocktail set-up, this strategy was found to result in a rather homogeneous population of functional and mature hepatocyte-like cells (Schwartz, 2002; Snykers, 2006). Here, 1 μ M TSA was added from 6 days onwards to both types of culture systems.

We found that TSA could improve and expedite the mesenchymal-to-hepatic transition, regardless of the set-up used. More specifically, TSA induced an earlier colonization of polygonal-shaped cells, improved the molecular hepatic phenotype and enhanced functional maturation. Yet, the degree of improvement differed

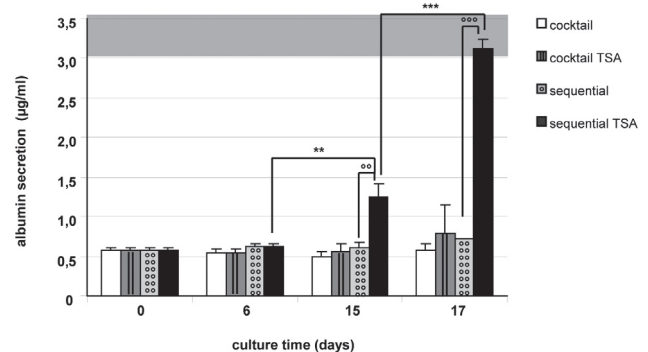


Fig. 4: ALB-secretion into the medium was measured by ELISA. rMPC were differentiated under sequential (+/- TSA) and cocktail (+/- TSA) conditions. The results are representative for 5 independent experiments. Grey area represents ALB-secretion levels measured in 2-days old monolayer cultures of primary rat hepatocytes, not exposed to TSA.

, *: ALB-secretion of rMPC cultivated under sequential-TSA conditions is significantly upregulated during culture time, $p < 0.01$ and $p < 0.001$ (Student's t-test).

°, °°: ALB-secretion of rMPC cultivated under sequential-TSA conditions is significantly higher than under sequential set-ups, $p < 0.01$ and $p < 0.001$ (Student's t-test).

among both culture systems. Under sequential conditions, TSA increased, prolonged and stabilised the overall expression level of typical hepatic proteins. Also, ALB-secretion was significantly

upregulated to levels comparable to those found in 2-days old monolayer cultures of adult hepatocytes (Vanhaecke, 2004a). In contrast, it was further found that, although TSA could considerably improve the hepatic maturation degree of cells cultured under cocktail conditions, their differentiated hepatic phenotype was not stabilized for long-term, nor was their hepatic functionality significantly improved. A possible explanation could be that *in vivo* a coordinated signalling between cells and the scaffold, (in)direct cell-cell contacts and stem cell-autonomous properties regulate in a proper spatio-temporal manner cell-fate decisions, including proliferation and differentiation (Theise, 2006; Shafritz, 2006; Moore, 2006). Therefore, next steps in the optimization of hepatic transdifferentiation strategies might comprise the introduction of (i) other cell types (co-culture) or their secretions (indirect co-culture) and (ii) natural scaffolds to mimic the microenvironment of liver stem cell niches *in vivo*.

Another issue that needs further investigation is the finding that TSA not only enhanced and prolonged expression of the mature markers ALB, CK18 and HNF1 α , but also of the immature markers HNF3 β and AFP. In particular APF-expression is known to drop down with progression of hepatic maturation, including the onset of ALB-expression (Shiojiri, 1981; Cascio, 1991). The continued AFP-expression in both cocktail-TSA and sequential-TSA conditions may indicate the formation of heterogeneous population of hepatic cells having different degrees of maturation or an atypical cellular hepatic phenotype. Additional analysis at the single cell level is needed to straighten this indistinctness.

In the present study, we have confirmed that TSA can significantly promote hepatic differentiation of adult bone marrow progenitor cells. Yet, further investigation is needed.

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