Thyroid hormones and tetrac: new regulators of tumour stroma formation via integrin $\alpha v \beta 3$

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Abstract

To improve our understanding of non-genomic, integrin $\alpha v \beta 3$ -mediated thyroid hormone action in tumour stroma formation, we examined the effects of triiodo-L-thyronine (T_3), L-thyroxine (T_4) and integrin-specific inhibitor tetrac on differentiation, migration and invasion of mesenchymal stem cells (MSCs) that are an integral part of the tumour's fibrovascular network. Primary human bone marrow-derived MSCs were treated with T_3 or T_4 in the presence of hepatocellular carcinoma (HCC) cell-conditioned medium (CM), which resulted in stimulation of the expression of genes associated with cancer-associated fibroblast-like differentiation as determined by qPCR and ELISA. In addition, T_3 and T_4 increased migration of MSCs towards HCC cell-CM and invasion into the centre of three-dimensional HCC cell spheroids. All these effects were tetrac-dependent and therefore integrin $\alpha v \beta 3$ -mediated. In a subcutaneous HCC xenograft model, MSCs showed significantly increased recruitment and invasion into tumours of hyperthyroid mice compared to euthyroid and, in particular, hypothyroid mice, while treatment with tetrac almost completely eliminated MSC recruitment. These studies significantly improve our understanding of the anti-tumour activity of tetrac, as well as the mechanisms that regulate MSC differentiation and recruitment in the context of tumour stroma formation, as an important prerequisite for the utilisation of MSCs as gene delivery vehicles.

Key Words

- mesenchymal stem cells
- thyroid hormones
- tetrac
- tumour stroma
- integrin $\alpha v \beta 3$

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Introduction

Tumours are composed of malignant tumour cells and the 'benign' stromal compartment that contains many distinct cell types, including endothelial cells, smooth muscle cells, cells of the immune system and pericytes/cancer-associated fibroblasts (CAFs) (Spaeth *et al.* 2008,

Dwyer *et al.* 2010). As the tumour stroma plays a key role in cancer progression with effects on tumour cell proliferation, angiogenesis and metastasis, it has become an attractive target for tumour therapy. Thus, understanding the biology of the tumour microenvironment is becoming

as important as knowledge of the neoplastic epithelial cells themselves.

Mesenchymal stem cells (MSCs) are multipotent non-haematopoietic progenitor cells that are characterised by their capacity to self-renew and differentiate into cells of connective tissue lineages such as bone, cartilage, muscle and adipose tissue (Klopp *et al.* 2007, Dwyer & Kerin 2010). In the course of tissue injury or during chronic inflammation, MSCs contribute to tissue remodelling by their mobilisation and subsequent recruitment to the site of injury (Klopp *et al.* 2007, Dwyer & Kerin 2010). Similarities between the wound-healing process and tumour stroma formation have led to the suggestion that tumours are 'wounds that do not heal' (Dvorak 1986). We and others have shown that MSCs are actively recruited to growing tumour stroma (Conrad *et al.* 2007, Zischek *et al.* 2009, Conrad *et al.* 2011, Knoop *et al.* 2011, Niess *et al.* 2011, Knoop *et al.* 2013) mediated by high local concentrations of inflammatory chemokines and growth factors (Ponte *et al.* 2007, Spaeth *et al.* 2008, Dwyer & Kerin 2010, Kholodenko *et al.* 2013). This tumour tropism constitutes the basis for the 'Trojan horse' approach, in which MSCs are used as shuttle vectors to deliver therapeutic agents into growing tumours (Dwyer & Kerin 2010, Dwyer *et al.* 2010, Dwyer *et al.* 2011, Dembinski *et al.* 2013, Zhu *et al.* 2014, Zhang *et al.* 2015), as we have successfully demonstrated in our previous work using the sodium iodide symporter (NIS) (Knoop *et al.* 2011, Knoop *et al.* 2013, Knoop *et al.* 2015) and herpes simplex virus type 1 thymidine kinase (HSV-TK) (Zischek *et al.* 2009, Conrad *et al.* 2011, Niess *et al.* 2011) as therapy genes in various tumour models.

Upon homing, MSCs act as important progenitors to subtypes that comprise the tumour stroma, including cells of the tumour vasculature and CAF-like cells (Barcellos-de-Souza *et al.* 2013). A CAF-like phenotype is defined by the following sets of markers: i) fibroblast surface markers; ii) indicators of tissue remodelling and invasion; iii) markers of angiogenesis; and iv) tumour promoting growth factors (Mishra *et al.* 2008, Spaeth *et al.* 2009, Kidd *et al.* 2012). While it is now well established that MSCs play a major role in supporting tumour vasculature and forming the tumour's fibrovascular network, the exact mechanisms behind their migration and differentiation in the tumour microenvironment and their impact on tumour progression are not yet fully understood.

Thyroid hormones 3,3',5-triiodo-L-thyronine (T_3) and L-thyroxine (T_4) are regulators of differentiation, growth and metabolism in most healthy tissues, but they have also

been proposed to play a critical role in tumour stroma formation by stimulation of angiogenesis, proliferation and inflammation (Luidens *et al.* 2010, Davis *et al.* 2011). These effects have been shown to be transduced by non-genomic mechanisms via a plasma membrane receptor for thyroid hormone on integrin $\alpha v \beta 3$ that was identified in 2005 by PJ Davis and colleagues (Bergh *et al.* 2005). Integrin $\alpha v \beta 3$ is predominantly concentrated in the plasma membrane of endothelial cells, vascular smooth muscle cells, osteoclasts and cancer cells, and is an attractive target for attempts to manipulate tumour cell proliferation and tumour-related neovascularisation (Davis *et al.* 2009). Tetraiodothyroacetic acid (tetrac), a deaminated T_4 derivative, is a specific inhibitor of thyroid hormone action at the integrin site (Bergh *et al.* 2005, Davis *et al.* 2011).

There is growing evidence that iodothyronines act as 'non-classical' pro-angiogenic modulators and induce neovascularisation. Pro-angiogenic actions of T_3 and T_4 are initiated at the hormone receptor on integrin $\alpha v \beta 3$, transduced by the MAPK/ERK pathway, and involve effects on basic fibroblast growth factor (FGF2), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) secretion (Luidens *et al.* 2010, Pinto *et al.* 2011). As an inhibitor of T_3/T_4 binding to $\alpha v \beta 3$, tetrac has been shown to inhibit angiogenic activity of thyroid hormones. In addition, $\alpha v \beta 3$ -mediated proliferative activity of T_3 and T_4 has been reported in various cancer cell lines. A recent uncontrolled observational study suggests that lowering of circulating T_4 levels is correlated with prolonged survival of terminal stage cancer patients (Hercbergs *et al.* 2015). In contrast, tetrac exerts a tumour growth-inhibitory effect based on the inhibition of T_3/T_4 effects, but also on agonist-independent direct anti-angiogenic and pro-apoptotic effects (Davis *et al.* 2011, Yalcin *et al.* 2013). In this context, an array of genes has been identified in human cancer cells that are regulated by tetrac through $\alpha v \beta 3$. This regulation includes stimulation of pro-apoptosis genes and downregulation of proto-oncogenes, cyclin genes, the *EGFR* gene, as well as genes in the Wnt/ β -catenin pathway, such as *CTNNA1* and *CTNNA2* (Davis *et al.* 2014).

MSCs are key players in the tumour microenvironment, and thyroid hormones have been shown to play a critical role in the regulation of tumour stroma formation. Therefore, in the current study we sought to investigate the non-genomic effects of T_3 , T_4 and tetrac on important aspects of MSC biology in the tumour milieu, such as differentiation, migration, recruitment and invasion as a critical prerequisite for the application of MSCs as gene delivery vehicles.

Materials and methods

Cell lines

Primary human bone marrow-derived CD34-negative MSCs (either prepared in house or provided by Apceth, Munich, Germany) were isolated and characterised according to the minimal criteria for MSCs released by the International Society for Cellular Therapy (Dominici *et al.* 2006). MSCs were used from passage 2 to 8 for experiments. The integrin $\alpha v\beta 3$ -negative human hepatocellular carcinoma (HCC) cell line HuH7 was authenticated and purchased from JCRB Cell Bank (Osaka, Japan) and passaged up to ten times in our laboratories. Both cell lines were grown in DMEM (Sigma–Aldrich) containing 10% (v/v) fetal bovine serum (FBS; FBS Superior, Biochrom/Merck Millipore, Berlin, Germany), 100 U/ml penicillin/100 µg/ml streptomycin (Sigma–Aldrich) and 2 mM L-glutamine (Sigma–Aldrich) and maintained at 37 °C in a humidified 5% (v/v) CO₂ atmosphere.

Thyroid hormone treatment *in vitro*

T_3 , T_4 and tetrac were dissolved in 0.1 N NaOH at 1 mg/ml. 25 µM (T_3 and T_4) or 100 µM (tetrac) stock solutions were prepared in sterile culture medium and stored in working aliquots at –20 °C protected from light. For the preparation of conditioned medium (CM), HuH7 were grown for 48 h to 80% confluence in DMEM containing 10% (v/v) charcoal-stripped FBS (csFBS; T_3 : <0.2 pg/ml, T_4 : 0.2–0.6 ng/dl). Supernatant was removed and centrifuged to eliminate cell debris. Prior to thyroid hormone treatment, MSCs were grown in DMEM/csFBS for 24 h. Medium was changed to 80% (v/v) DMEM/csFBS and 20% (v/v) HuH7 CM for differentiation studies or DMEM/csFBS for migration and invasion studies and supplemented with 1 nM T_3 or 1 µM T_4 with and without 100 nM tetrac for 24 h. In a pilot study, ranges of 1–100 nM T_3 as well as 1–1000 nM T_4 were tested and optimal concentrations of 1 nM total T_3 (~30 pM free T_3) and 1 µM total T_4 (~1.5 nM free T_4) were used for further experiments.

Flow cytometry

For flow cytometry, MSCs and HuH7 were harvested with EDTA (Sigma–Aldrich) and washed with PBS (Sigma–Aldrich) supplemented with 10% (v/v) FBS (FACS buffer). Cells were incubated with a mouse monoclonal anti-integrin $\alpha v\beta 3$ antibody (2 µg/10⁶ cells; Abcam, Cambridge, UK) for 45 min on ice, washed with FACS buffer and then

incubated with a Cy3-conjugated polyclonal donkey anti-mouse antibody (2 µg/10⁶ cells; Jackson, Baltimore, MD, USA) for 30 min on ice. Cells incubated without the primary antibody served as negative control. Analysis was performed in the FL2 channel of a BD Accuri C6 flow cytometer using Cflow software (BD Biosciences, Franklin Lakes, NJ, USA). 10⁵ events were recorded for each sample. Cell debris was excluded from analysis by appropriate gating.

Quantitative real-time PCR

Total RNA from MSCs was prepared using the RNeasy Mini Kit with QIAshredder (Qiagen). RT and quantitative real-time PCR were performed as described previously (Knoop *et al.* 2013) and run in a Mastercycler ep gradient S PCR cyclor (Eppendorf, Hamburg, Germany). Relative expression levels were calculated from $\Delta\Delta C_t$ values normalised to internal β -actin and 18S rRNA. Primers are listed in Table 1.

ELISA

Supernatant from MSCs was removed, centrifuged and stored at –80 °C until assayed for EGF, FGF2, hepatocyte growth factor (HGF), interleukin 6 (IL6), stromal-derived factor 1 (SDF-1), transforming growth factor beta1 (TGF- β 1), thrombospondin 1 (TSP1) and VEGF concentrations using the respective DuoSet ELISA kit (R&D Systems, Abingdon, UK). Contributions from medium and HuH7 CM were subtracted.

Migration assay

MSC migration was analysed using the μ -slide Chemo-taxis^{3D} system from ibidi (Martinsried, Germany). MSCs were seeded in collagen I (0.3 × 10⁶ cells/ml) and subjected to a gradient between serum-free unconditioned medium and serum-free HuH7 CM. Both media and the collagen gel contained either no thyroid hormone, 1 nM T_3 or 1 µM T_4 with and without 100 nM tetrac. Chemotaxis was monitored by time-lapse microscopy over a 24-h period on a Leica DM IL microscope (Leica Microsystems, Wetzlar, Germany). Pictures were taken every 15 min with a Jenoptik ProgRes CCD camera (Jenoptik, Jena, Germany) controlled by the open-source ImageJ (NIH, Bethesda, MD, USA) plug-in μ Manager (Edelstein *et al.* 2010). Twenty-five randomly selected cells were manually tracked with the ImageJ plug-in Manual Tracking (Fabrice Cordelières, Orsay, France) and analysed with Chemotaxis and Migration Tool Software (ibidi). The migratory behaviour of cells was quantified by forward migration index (FMI),

Table 1 qPCR primer sequences

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
ACTA2 (α -SMA)	ACCCACAATGTCCCCATCTA	GAAGGAATAGCCACGCTCAG
ACTB (β -actin)	AGAAAATCTGGCACCACACC	TAGCACAGCCTGGATAGCAA
CXCL12 (SDF-1)	AGAGCCAACGTCAAGCATCT	CTTTAGCTTCGGGTCAATGC
DES (desmin)	AAGCTGCAGGAGGAGATTCA	GGCAGTGAGGTCTGGCTTAG
EGF	CAGGGAAGATGACCACCACT	CAGTTCCCACTCTCAGGT
FAP	GGTCTCCCAACAAAGGATGA	CATTGCCTGGAAATCCACTT
FGF2	GGAGAAGAGCGACCTCAC	AGCCAGGTAACGGTTAGCAC
HGF	CTGGTTCCCTTCAATAGCA	CTCCAGGGCTGACATTTGAT
IL6	TACCCCAAGGAGAAGATTCC	TTTTCTGCCAGTGCTCTTT
MMP3 (SL-1)	GCAGTTTGCTCAGCCTATCC	GAGTGTCTGGAGTCCAGCTTC
R18s	CAGCCACCCGAGATTGAGCA	TAGTAGCGACGGGCGGTGTG
S100A4 (FSP1)	CCCTGGATGTGATGGTGTC	CGATGCAGGACAGGAAGACA
TGFB1 (TGF- β 1)	CAGCACGTGGAGCTGTACC	AAGATAACCACTCTGGCGAGTC
THBS1 (TSP1)	TTGTCTTTGGAACCAACCA	CTGGACAGCTCATCACAGGA
TNC	TTCAGTGAGCTGACTGTGG	TAGGGCAGCTCATGTCACTG
VEGF	CTACCTCCACCATGCCAAGT	ATGATTCTGCCCTCTCCTT

a measure of the efficiency of the migration of cells in relation to the CM gradient and centre-of-mass (CoM) displacement that is calculated from the averaged point of all cell endpoints.

Spheroid invasion assay

Invasion assays were performed as described previously (Huiskens *et al.* 2004, Pitrone *et al.* 2013, Gualda *et al.* 2014, Rühland *et al.* 2015). In brief, spheroids of 200–300 μ m in diameter were grown from HuH7 cells. Single spheroids were rolled in 2.5×10^4 thyroid hormone-treated, Cell-Tracker Green CMFDA- (5-chloromethylfluorescein diacetate, Life Technologies) labelled MSCs for 2 h, washed and incubated for further 24 h. Spheroids were fixed in 4% formalin, embedded in 1% (w/v) 2-hydroxyethylagarose (Roth, Karlsruhe, Germany) containing green fluorescent microspheres (F-XC 50 Estapor, Merck; 1:50 000 dilution) and aspirated into a glass capillary. Imaging was performed with a 488 nm laser (2 mW laser power, 120 ms exposure time; Cube, Coherent, Santa Clara, CA, USA) and an sCMOS camera (Orca-flash 4.0 V2, Hamamatsu, Hamamatsu City, Japan) from five different angles equally spaced over 360°, controlled via the FIJI μ Manager plug-in (Edelstein *et al.* 2010). The subsequent registration of beads and fusion of images was performed using SPIM open-source software on FIJI (Preibisch *et al.* 2010). Signals from CMFDA-labelled MSCs and the autofluorescent spheroid were detected and segmented using the FIJI 3D object counter plugin (Bolte & Cordelières 2006). Invasion depths were quantified by measurement of distances from the centre of each MSC to the border of the spheroid using the FIJI 3D manager plugin (Ollion *et al.* 2013).

Animals

Male CD1 nu/nu mice from Charles River (Sulzfeld, Germany) were maintained under specific pathogen-free conditions with access to standard nude mouse diet (ssniff, Soest, Germany) and water *ad libitum*. Animals were allowed to acclimatise for 1 week prior to the start of treatments. Experiments were conducted with animals between 5 and 12 weeks of age. Thyroid hormone status was regularly monitored in serum samples as described previously (Schmohl *et al.* 2015). The experimental protocol was approved by the regional governmental commission for animals (Regierung von Oberbayern, Munich, Germany).

MSC recruitment to tumours *in vivo*

HuH7 xenograft tumours were established by s.c. injection of 3.5×10^6 HuH7 cells in 100 μ l PBS into the flank region. Three days later, animals received drinking water supplemented with 0.02% (w/v) 2-mercapto-1-methylimidazole (MMI; Sigma–Aldrich), 1% (w/v) sodium perchlorate (Sigma–Aldrich) and 0.3% (w/v) saccharin (Sigma–Aldrich) (Groba *et al.* 2013) to induce hypothyroidism in order to generate the same baseline thyroid hormone levels for all groups. Three weeks later, once small tumours (<3 mm) were visible, mice were randomly assigned to different groups by daily i.p. injections. The hyperthyroid group ($n=8$) received 100 ng/g body weight $L-T_4$ (Sigma–Aldrich), the euthyroid group 20 ng/g body weight $L-T_4$ with ($n=8$) or without ($n=7$) 10 μ g/g body weight tetrac (Sigma–Aldrich), hypothyroid animals ($n=8$) received saline only. Eighteen days later (tumour volume

$\sim 500 \text{ mm}^3$), 5×10^5 MSCs cytoplasmically labelled with $20 \mu\text{M}$ CMFDA for 30 min were injected via the tail vein in $500 \mu\text{l}$ PBS. Seventy-two hours later, mice were sacrificed and tumours were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Alphen aan den Rijn, the Netherlands), snap-frozen on dry ice and stored at -80°C .

Immunofluorescence microscopy

Tissue sections were fixed in methanol/acetone, counterstained with $5 \mu\text{g/ml}$ bisbenzimidazole Hoechst 33258 (Sigma-Aldrich), mounted with Dako Fluorescence Mounting Medium (Dako, Carpinteria, CA, USA) and imaged at $10\times$ magnification on an Axiovert 135 TV fluorescence microscope equipped with an AxioCam MRm CCD camera and AxioVision Rel. 4.8 Software (Carl Zeiss, Munich, Germany). Four to seven visual fields in perivascular regions were recorded per animal and MSC recruitment was quantified as a percentage of CMFDA-positive area using ImageJ software (NIH, Bethesda, MD, USA).

Statistics

All *in vitro* experiments were performed at least in triplicate. *In vivo* data were generated in two independent experiments. Values are reported as mean $\pm$ S.E.M. or mean fold change $\pm$ S.E.M. Statistical significance was tested by two-tailed Student's *t*-test or, for invasion assays, by Kruskal–Wallis test. *P*-values <0.05 were considered significant ($*P<0.05$; $**P<0.01$; $***P<0.001$).

Results

Expression of integrin $\alpha v\beta 3$ is absent on HuH7 cells and not influenced by thyroid hormones T_3 and T_4 or tetrac on MSCs

Absence of integrin $\alpha v\beta 3$ expression on HuH7, as well as presence of $\alpha v\beta 3$ on MSCs, was confirmed by flow cytometry (Fig. 1). Treatment of MSCs with 1 nM T_3 or $1 \mu\text{M}$ T_4 in the presence or absence of tetrac (100 nM) or tetrac alone did not alter expression levels of the integrin (Fig. 1).

Thyroid hormones T_3 and T_4 increase CAF-like differentiation of MSCs *in vitro*

Twenty-four-hour stimulation of MSCs with HuH7 CM in the presence of 1 nM T_3 or $1 \mu\text{M}$ T_4 resulted in significantly

Figure 1

Evaluation of integrin $\alpha v\beta 3$ expression on HuH7 and MSCs. MSCs were treated with HuH7 CM alone or in the presence of 1 nM T_3 or $1 \mu\text{M}$ T_4 with or without 100 nM tetrac, or in the presence of tetrac alone for 24 h. HuH7 and MSCs were analysed for integrin $\alpha v\beta 3$ expression by flow cytometry. A representative image from three experiments is shown.

increased mRNA levels for genes associated with a CAF-like phenotype. Upon T_3 treatment, surface marker fibroblast-specific protein 1 (FSP1; *S100A4*), markers of angiogenesis desmin and VEGF, as well as tumour-promoting growth factors EGF, FGF2, HGF, IL6, SDF-1 and TGF- $\beta 1$, were expressed at significantly higher mRNA levels as compared to control cells treated with HuH7 CM alone. Surface marker fibroblast activation protein (FAP), indicators of tissue remodelling and invasion tenascin-C (TN-C) and TSP1 (*THBS1*), as well as the angiogenesis marker alpha-smooth muscle actin (α -SMA; *ACTA2*), showed the same trend that was, however, not significant (Fig. 2A). T_4 stimulation led to significant increases in mRNA expression for FAP, TN-C, TSP1, α -SMA and desmin, as well as FGF2, IL6, SDF-1 and TGF- $\beta 1$, while the same, albeit not significant, trends were seen for FSP1, VEGF, EGF and HGF (Fig. 2B). The marker of invasion and remodelling stromelysin-1 (SL-1/MMP3) was not detected for any of the conditions tested (not shown). Effects seen at the mRNA level were confirmed at the protein level for TSP1, VEGF, IL6, SDF-1 and TGF- $\beta 1$ by ELISA (Fig. 2C). Protein levels for EGF, FGF2 and HGF remained below the detection limit of the ELISA kits used for all treatment conditions. The T_3 - and T_4 -triggered increases in gene and protein concentrations were found to be tetrac-dependent and therefore $\alpha v\beta 3$ -mediated. No significant changes were observed when cells were treated with tetrac alone (data not shown).

Thyroid hormones T_3 and T_4 increase migration of MSCs towards tumour signals

MSCs subjected to a gradient between HuH7 CM and serum-free medium showed directed chemotaxis towards CM with significantly increased FMI and CoM

Figure 2

Expression of CAF-like markers in MSCs is stimulated by thyroid hormones T_3 and T_4 via integrin $\alpha v \beta 3$. MSCs were treated with HuH7 CM alone (grey line) or in the presence of 1 nM T_3 or 1 μ M T_4 with or without 100 nM tetrac, or in the presence of tetrac alone for 24 h. Expression of CAF-like

markers was assessed at the mRNA level by qPCR ((A) T_3 , (B) T_4) and at the protein level by ELISA (C). Results are expressed as mean fold change compared to control cells treated with HuH7 CM only ($n=4$; two-tailed Student's t -test: * $P<0.05$; ** $P<0.01$; *** $P<0.001$).

displacement along the gradient of 0.227 ± 0.041 ($P<0.001$) and $84.7 \pm 18.8 \mu$ m ($P<0.001$) respectively (Fig. 3B, H and I) compared to untreated cells not subjected to a gradient that showed basal random chemokinesis (FMI -0.060 ± 0.025 and CoM $-26.8 \pm 11.3 \mu$ m; Fig. 3A). This set-up was used to analyse the effects of T_3 , T_4 and tetrac on the migratory behaviour of MSCs towards HuH7 CM. MSCs were pretreated with thyroid hormone with or without tetrac for 24 h and kept under the same T_3/T_4 /tetrac concentrations throughout the chemotaxis assay. Migration of MSCs towards CM was significantly increased upon treatment with 1 nM T_3 (FMI: 0.383 ± 0.049 ; $P<0.05$ and CoM $167.3 \pm 21.2 \mu$ m; $P<0.05$; Fig. 3D, H and I) or 1 μ M T_4 (FMI: 0.345 ± 0.032 ; $P<0.05$ and CoM $160.0 \pm 12.8 \mu$ m; $P<0.01$; Fig. 3F, H and I) compared to untreated control cells. Additional treatment with 100 nM tetrac inhibited the effects of T_3 and T_4 respectively on MSC migration (T_3 +tetrac: FMI: 0.219 ± 0.023 ; $P<0.05$ vs T_3 and CoM $102.5 \pm 13.8 \mu$ m; $P<0.05$ vs T_3 ; Fig. 3E, H and I; T_4 +tetrac: FMI: 0.261 ± 0.029 ; $P=0.055$ vs T_4 and CoM $127.9 \pm 19.2 \mu$ m; $P=0.16$ vs T_4 ; Fig. 3G, H and I). Cells treated with tetrac alone showed no significant change in migratory behaviour compared to control cells (FMI: 0.275 ± 0.049 ; $P=0.47$ and CoM $106.3 \pm 19.4 \mu$ m; $P=0.44$; Fig. 3C).

Thyroid hormones T_3 and T_4 increase invasion of MSCs into tumour cell spheroids

The invasive capacity of MSCs was evaluated in a three-dimensional HuH7 spheroid model. MSCs were pretreated with 1 nM T_3 or 1 μ M T_4 in the presence or absence of 100 nM tetrac before attaching to spheroids. Both after T_3 (Fig. 4B) and T_4 stimulation (Fig. 4D), MSCs showed deeper invasion (T_3 : $34.5 \pm 7.42 \mu$ m; $P=0.15$ and T_4 : $29.00 \pm 1.37 \mu$ m; $P<0.01$) into the centre of spheroids compared to untreated control cells ($21.83 \pm 1.79 \mu$ m; Fig. 4A and F). Cells treated with T_3 or T_4 in the presence of tetrac (T_3 +tetrac: $9.00 \pm 1.78 \mu$ m; $P<0.05$ vs T_3 and T_4 +tetrac: $22.25 \pm 1.55 \mu$ m; $P<0.01$ vs T_4) showed inhibition of invasion and remained at the surface (Fig. 4C, E and F). In some cases, especially after T_3 treatment, invaded cells clustered inside the spheroid and could not be resolved to single cells. Clusters (marked by asterisks in Fig. 4F) were therefore counted as single cells.

T_4 increases tumour migration and invasion *in vivo*

To assess the effect of thyroid hormone status and tetrac on MSC recruitment and invasion into tumours *in vivo*, nude mice harbouring HuH7 xenografts were subjected to different thyroid hormone treatments (euthyroid,

Figure 3

Migration of MSCs towards tumour cell CM is increased upon T_3 and T_4 treatment mediated by integrin $\alpha\text{v}\beta_3$. (A) Representative trajectories of untreated MSCs without gradient influence. (B, C, D, E, F and G) Representative trajectories of MSCs treated with 1 nM T_3 or 1 μM T_4 with or without 100 nM tetrac or with tetrac alone for 24 h migrating along a

linear gradient of HuH7 CM over 24 h compared to untreated control cells from six independent experiments. Centres of mass are indicated by red dots. (H, I) Quantification of chemotaxis parameters FMI and CoM (two-tailed Student's *t*-test: * $P < 0.05$; ** $P < 0.01$).

hyperthyroid, hypothyroid and euthyroid+tetrac; for serum thyroid hormone concentrations see [Supplementary Figure 1](#), see section on [supplementary data](#) given at the end of this article) and, 72 h before sacrifice, were systemically injected with 5×10^5 CMFDA-labelled MSCs. Immunofluorescence microscopy ([Fig. 5A](#)) revealed significantly increased recruitment of MSCs to perivascular regions in the tumour and invasion into the surrounding tumour tissue in hyperthyroid mice (CMFDA positive area

$25.7 \pm 2.1\%$; $P < 0.001$; [Fig. 5B](#)) compared to euthyroid mice (CMFDA positive area $12.2 \pm 1.0\%$; [Fig. 5B](#)). In hypothyroid mice, recruitment and invasion were strikingly reduced (CMFDA positive area $6.7 \pm 0.7\%$; $P < 0.001$; [Fig. 5B](#)). Treatment of euthyroid mice with tetrac resulted in an additional reduction in MSC recruitment, revealing only a thin margin of MSCs surrounding blood vessels and no detectable tissue invasion (CMFDA positive area $2.0 \pm 0.2\%$; $P < 0.001$; [Fig. 5B](#)). Neither thyroid hormone

Figure 4

Deeper invasion of MSCs into 3D HuH7 spheroids upon T_3 and T_4 treatment is reversed upon additional treatment with tetrac. (A, B, C, D and E) Representative images of CMFDA-labelled MSCs (green) treated with 1 nM T_3 or 1 μ M T_4 with or without 100 nM tetrac for 24 h invading into HuH7

spheroids (blue). (F) Quantification of invasion depths in distance from surface of the spheroid ($n=3$; Kruskal–Wallis test: ** $P<0.01$). Cell clusters are indicated by asterisks.

status nor tetrac treatment had a significant effect on tumour growth in this model (data not shown).

Discussion

Based on the fact that both MSCs and thyroid hormones impact crucial steps of tumour stroma formation (Davis *et al.* 2011, Barcellos-de-Souza *et al.* 2013), we investigated the regulation of MSC biology in the tumour microenvironment by non-genomic effects of T_3 , T_4 and tetrac. To distinguish between the effects on MSCs/stroma biology as opposed to direct effects on the tumour, we selected an $\alpha v \beta 3$ -negative HCC as an experimental model (Jin *et al.* 2012).

First, we addressed the question as to whether thyroid hormones can enhance the CAF-like phenotype that MSCs acquire when exposed to tumour cell-CM (Mishra *et al.* 2008, Spaeth *et al.* 2009, Barcellos-de-Souza *et al.* 2013). Compared to MSCs treated with HuH7 CM alone, upon additional treatment with T_3 or T_4 , we observed an overall enhanced expression of i) fibroblast surface markers FAP and FSP; ii) indicators of tissue remodelling and invasion TN-C and TSP1; iii) proteins associated with angiogenesis α -SMA, desmin and VEGF; and iv) tumour-promoting growth factors/chemokines EGF, FGF2, HGF, IL6, SDF-1 and TGF- β 1, suggesting a T_3 / T_4 -enhanced CAF-like differentiation of MSCs mediated through $\alpha v \beta 3$ as shown by inhibition of these effects by tetrac.

In line with our observations, transduction of the thyroid hormone signal via integrin $\alpha v \beta 3$ was shown to lead to transcription of angiogenesis-relevant genes *FGF2* and *VEGF* in the chorioallantoic membrane (CAM) model, as well as in heart and T-cell lymphoma (Luidens *et al.* 2010, Cayrol *et al.* 2015). A direct effect of thyroid hormones on inflammatory mediators such as IL6 has

been tentatively proposed based on the inhibition of pro-inflammatory cytokines by the iodothyronine antagonist tetrac (Hercbergs *et al.* 2012). Integrin $\alpha v \beta 3$ has been shown to engage in crosstalk with tyrosine kinase growth factor receptors such as EGF receptor, FGF receptor, platelet-derived growth factor (PDGF) receptor and VEGF receptor, which are all expressed by MSCs (Spaeth *et al.* 2008, Davis *et al.* 2011, Hercbergs *et al.* 2012). Explanations for the changes in the expression levels of markers of CAF-like differentiation shown here could involve binding of CM-derived growth factors to their respective receptors on MSCs or autocrine stimulation by growth factors secreted by MSCs in response to CM, with potentiation of the signal through integrin $\alpha v \beta 3$ crosstalk. A further indirect effect could involve an enhanced expression of hypoxia-inducible factor 1 alpha (HIF-1 α) that was shown to be increased upon T_3 binding to integrin $\alpha v \beta 3$ transduced by the phosphoinositide 3-kinase (PI3K) pathway, which in turn stimulates the secretion of HIF-responsive genes, such as *VEGF*, *FGF2*, *IL6*, *SDF1* and *TGFB1* (Davis *et al.* 2011, Hercbergs *et al.* 2012). However, the exact mechanisms behind the synergistic effects of CM and T_3 / T_4 on MSC differentiation remain to be elucidated.

In addition, we observed enhanced chemotaxis of MSCs towards tumour signals and invasion into three-dimensional HCC cell spheroids under thyroid hormone stimulation, mediated by $\alpha v \beta 3$. Balzan *et al.* (2013) reported similar effects on microvascular endothelial cells in a wound-healing assay with increased migration after T_3 or T_4 stimulation that was abolished by treatment with tetrac. Studies on multiple myeloma cells and bone marrow aspirates from multiple myeloma patients by Cohen *et al.* (2014) illustrate thyroid hormone-dependent, $\alpha v \beta 3$ -mediated regulation of cell migration. Furthermore,

Figure 5

Thyroid hormone status significantly influences MSC recruitment into tumours *in vivo*. In a HuH7 tumour xenograft model, mice were injected daily with 20 ng/g body weight $L-T_4$ (euthyroid; $n=7$), 100 ng/g body weight $L-T_4$ (hyperthyroid; $n=8$), saline (hypoathyroid; $n=8$) or 20 ng/g body weight $L-T_4$ + 10 μ g/g body weight tetrac (euthyroid + tetrac; $n=8$). 72 h before sacrifice, 5×10^5 CMFDA-labelled MSCs were injected i.v. Frozen

tumour sections were counterstained with Hoechst dye and imaged by fluorescence microscopy. (A) One representative image is shown per group. Magnification: $10\times$, scale bar = 100 μ m. (B) Quantification of MSC recruitment to tumours depicted as mean values of CMFDA positive area in % for each group (two-tailed Student's *t*-test: *** $P < 0.001$).

there is growing evidence that thyroid hormones increase the motility of $\alpha v\beta 3$ -expressing immune cells, and, possibly by similar mechanisms, nerve cell migration (De Vito *et al.* 2012). An array of growth factors, including PDGF, insulin-like growth factor 1 (IGF1), HGF, EGF and VEGF, as well as chemokines, such as RANTES/CCL5, CCL22 and, to a lower extent, SDF-1/CXCL12 and their respective receptors, have been implicated in MSC migration (Ponte *et al.* 2007, Spaeth *et al.* 2008) and many of these have been shown in this and other studies to be regulated by thyroid hormones via differential gene expression and receptor crosstalk (Davis *et al.* 2011, Herbergs *et al.* 2012).

Modulation of recruitment and engraftment efficiency of MSCs is of clinical interest, in settings of tissue regeneration, in the context of general tumour growth and the emerging field of MSC-based gene delivery in

cancer therapy (Knoop *et al.* 2011, Knoop *et al.* 2013, Uchibori *et al.* 2014). As the tumour stroma plays such a key role in tumour growth, angiogenesis and metastatic potential, it has become an important target for tumour therapy. Due to their natural tropism for solid tumours and metastases, as well as their relative ease of engineering and expansion *in vitro*, MSCs are excellent gene delivery vehicles to target tumour environments. In a series of previous studies we demonstrated active homing of HSV-TK-transduced MSCs into pancreatic, breast and liver cancer tumour stroma that led to a significant reduction in tumour growth and, depending on the targeting strategy used, reduced incidence of metastases after application of ganciclovir (Zischek *et al.* 2009, Conrad *et al.* 2011, Niess *et al.* 2011). In more recent studies, we have shown that MSCs transfected with NIS under control of the CMV promoter or the tumour stroma-specific RANTES/CCL5

promoter are actively recruited to experimental HCC as well as disseminated colon cancer liver metastases and induce anti-tumour effects after application of ^{131}I or ^{188}Re based on NIS-mediated radioiodine accumulation (Knoop et al. 2011, Knoop et al. 2013). However, full therapeutic potential can only be exploited when MSCs are efficiently recruited to their site of action. Several approaches have been used to increase MSC survival, migration and differentiation, including the application of growth factors and overexpression of stem cell regulatory genes (Chen et al. 2013). In agreement with our *in vitro* data, we observed a significant impact of thyroid hormone status on MSC recruitment and invasion in an $\alpha\text{v}\beta 3$ -negative HCC xenograft mouse model. In hyperthyroid animals, both the recruitment of MSCs and their subsequent invasion into tumours was markedly enhanced in comparison with euthyroid and in particular hypothyroid mice that showed low levels of MSC recruitment and almost no invasion. These data suggest that MSC conditioning with T_3 or T_4 and/or T_3 or T_4 treatment of patients could serve as an effective tool to enhance MSC migration and engraftment in tissue-engineering and gene-delivery approaches.

At the same time, it should be taken into account that the stimulatory effect of iodothyronines, shown in our study, on the differentiation of MSCs towards CAFs that are known to support a microenvironment that drives tumour progression and metastasis, could enhance tumour growth (Franco et al. 2010). However, depending on the approach used, MSC-based cancer gene therapy can include the destruction of exogenously applied engineered MSCs in the context of therapy, which is likely to overcome any endogenous tumour-promoting effects of adoptively applied MSCs. This was demonstrated in our previous work using MSCs as delivery vehicles for tumour-selective NIS gene delivery, showing no tumour growth-promoting effects in subcutaneous HuH7 or hepatic colon cancer metastasis xenograft mouse models where a significant therapeutic effect of ^{131}I or ^{188}Re was seen (Knoop et al. 2011, Knoop et al. 2013). Moreover, we did not observe an effect of thyroid hormone status or tetrac treatment on the growth of the HCC xenograft model used in this study, which we attribute to the absence of integrin $\alpha\text{v}\beta 3$ on HuH7 cells and also to the short time frame for the assessment of tumour stroma-mediated effects on tumour growth in our experimental setting.

Further, we observed a dramatic effect of tetrac treatment in euthyroid mice that led to a nearly complete abolishment of MSC recruitment into the tumour. Tetrac also reversed effects of T_3 and T_4 on CAF marker gene expression, migration and invasion *in vitro*. While tetrac is known to exert low-grade thyromimetic effects

intracellularly (Moreno et al. 2008), it was shown to have anti-tumour effects *in vitro* and *in vivo* in various cancer models via modulation of tumour cell proliferation, apoptosis and angiogenesis by T_3/T_4 antagonistic action mediated through integrin $\alpha\text{v}\beta 3$ (Yalcin et al. 2009, Davis et al. 2011). Further anti-tumour activity of tetrac includes the suppression of invasiveness/metastasis, increased radio- and chemosensitisation and antagonism of inflammation (Davis et al. 2011, Herbergs et al. 2012, Yalcin et al. 2013, Davis et al. 2014). These effects are thought to be largely mediated by $\alpha\text{v}\beta 3$ expressed on the tumour cells themselves. Using an $\alpha\text{v}\beta 3$ -negative tumour model, our data suggest a novel mechanism by which tetrac can exert anti-tumoural effects, not by targeting the cancer cells *per se*, but by targeting the tumour stroma via MSCs as important stromal progenitor cells. Besides presenting an additional aspect of tetrac's anti-tumour action, this effect opens the prospect of using tetrac as therapy agent irrespective of integrin $\alpha\text{v}\beta 3$ expression on tumour cells.

In conclusion, our data suggest that thyroid hormones T_3 and T_4 have a profound effect on the biology of MSCs in the tumour microenvironment through stimulation of CAF-like differentiation as well as migration and invasion *in vitro*. Tetrac-dependency demonstrates that these effects are largely mediated through non-genomic mechanisms via integrin $\alpha\text{v}\beta 3$. *In vivo*, the thyroid status had a dramatic impact on MSC recruitment and tumour invasion with increased recruitment in the hyperthyroid state that was significantly decreased in the hypothyroid state and almost abolished upon treatment with tetrac. These studies enhance our understanding of the critical role of T_3 and T_4 in the regulation of MSC differentiation and migration in the context of tumour stroma formation and stroma-targeted cancer therapy, as well as the molecular mechanisms of the anti-tumour activity of tetrac.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-15-0245>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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