Thyroid hormones and tetrac: new regulators of tumour stroma formation via integrin αvβ3

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Abstract

To improve our understanding of non-genomic, integrin αvβ3-mediated thyroid hormone action in tumour stroma formation, we examined the effects of triiodo-L-thyronine (T3), L-thyroxine (T4) 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 T3 or T4 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, T3 and T4 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 αvβ3-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 αvβ3

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 of 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-1-thyronine (T₃) and 1-thyroxine (T₄) 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 αvβ3 that was identified in 2005 by PJ Davis and colleagues (Bergh et al. 2005). Integrin αvβ3 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 T₄ 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 T₃ and T₄ are initiated at the hormone receptor on integrin αvβ3, 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 T₃/T₄ binding to αvβ3, tetrac has been shown to inhibit angiogenic activity of thyroid hormones. In addition, αvβ3-mediated proliferative activity of T₃ and T₄ has been reported in various cancer cell lines. A recent uncontrolled observational study suggests that lowering of circulating T₄ levels is correlated with prolonged survival of terminal stage cancer patients (Hercebergs et al. 2015). In contrast, tetrac exerts a tumour growth-inhibitory effect based on the inhibition of T₃/T₄ 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 αvβ3. 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 stoma formation. Therefore, in the current study we sought to investigate the non-genomic effects of T₃, T₄ 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 αvβ3-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

T₃, T₄ and tetric were dissolved in 0.1 N NaOH at 1 mg/ml. 25 μM (T₃ and T₄) or 100 μM (tetric) 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; T₃ for 48 h to 80% confluence in DMEM containing 10% (v/v) charcoal-stripped FBS (csFBS; T₃: <0.2 pg/ml, T₄: 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 T₃ or 1 μM T₄ with and without 100 nM tetric for 24 h. In a pilot study, ranges of 1–100 nM T₃ as well as 1–1000 nM T₄ were tested and optimal concentrations of 1 nM total T₃ (~30 pM free T₃) and 1 μM total T₄ (~1.5 nM free T₄) 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 αvβ3 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 cycler (Eppendorf, Hamburg, Germany). Relative expression levels were calculated from ∆∆Ct 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-β1), 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 Chemotaxis 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 conditioned medium and serum-free HuH7 CM. Both media and the collagen gel contained either no thyroid hormone, 1 nM T₃ or 1 μM T₄ with and without 100 nM tetric. 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 μManager (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),
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 (Huisken et al. 2004, Pitrone et al. 2013, Gualda et al. 2014, Rühland et al. 2015). In brief, spheroids of 200–300 μm diameter were grown from HuH7 cells. Single spheroids were rolled in 2.5 x 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 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 & Cordelieres 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 x 10^6 HuH7 cells in 100 μl 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 1-T4 (Sigma–Aldrich), the euthyroid group 20 ng/g body weight 1-T4 with (n = 8) or without (n = 7) 10 μg/g body weight tetrac (Sigma–Aldrich), hypothyroid animals (n = 8) received saline only. Eighteen days later (tumour volume

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**Table 1 qPCR primer sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTA2 (α-SMA)</td>
<td>ACCCAAAATTGCCTCCCATCTA</td>
<td>GAAGGAATTGCCACGGTCA</td>
</tr>
<tr>
<td>ACTB (β-actin)</td>
<td>AGAAAAATCTGGACACACACC</td>
<td>TAGCAACAGCTTGCAGTFAAGCAA</td>
</tr>
<tr>
<td>CXCL12 (SDF-1)</td>
<td>AAGCCCAAGGTCAAGACCTCT</td>
<td>CTTAGCTTCGGGTCTACAG</td>
</tr>
<tr>
<td>DES (desmin)</td>
<td>GAGGGAGAGTACCCACC</td>
<td>GGCAGTTGGCTGCTTGA</td>
</tr>
<tr>
<td>FAP</td>
<td>GGCTTCCAACAAACAGGTA</td>
<td>CATTGCTCTGAGAAATCCGT</td>
</tr>
<tr>
<td>FGF2</td>
<td>GGAAGAAGCCAGCACTCAC</td>
<td>AGCCAGTAAACCTGGTACG</td>
</tr>
<tr>
<td>HGF</td>
<td>TGGTCCCTCCTTCCATAGCA</td>
<td>CTCGACGGGTCAACATTGTG</td>
</tr>
<tr>
<td>IL6</td>
<td>TACCCCCAGGAGAAGAAGTGC</td>
<td>TTCTCCAGGTAGTGGTCC</td>
</tr>
<tr>
<td>MMP3 (SL-1)</td>
<td>AGAGCCAACGTCAAGCATC</td>
<td>GAGGTGTTGGTGCTACCTATC</td>
</tr>
<tr>
<td>RTB4</td>
<td>CACACCCACGAGTACAGGCA</td>
<td>TAGTACCGGACGCGCGGTGTG</td>
</tr>
<tr>
<td>S100A4 (FSP1)</td>
<td>CCTGGATGTGATGTGTC</td>
<td>CTGGCAGGACGAAAGAGCA</td>
</tr>
<tr>
<td>TGFβ1 (TGF-β1)</td>
<td>CAGACGCTGGAGCTGTACC</td>
<td>AAAGTAAACCTCTGTGCGAGTC</td>
</tr>
<tr>
<td>TβHS1 (TSP1)</td>
<td>TGTCTTTGGAAACCCACCA</td>
<td>CTGGAAGCTCAGCATCACAGGA</td>
</tr>
<tr>
<td>TNC</td>
<td>TACACTGGAGCTGACTGTGG</td>
<td>TAGGGAGCTCAGTGCACCTG</td>
</tr>
<tr>
<td>VEGF</td>
<td>CTACCCTACCATTGCCAGT</td>
<td>AGTATTCCGCTCCCTCCTT</td>
</tr>
</tbody>
</table>

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~ 500 mm$^3$), $5 \times 10^5$ MSCs cytoplasmically labelled with 20 $\mu$m CMFDA for 30 min were injected via the tail vein in 500 $\mu$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 $-80^\circ$C.

**Immunofluorescence microscopy**

Tissue sections were fixed in methanol/acetone, counter-stained with 5 $\mu$/ml bisbenzimide Hoechst 33258 (Sigma–Aldrich), mounted with Dako Fluorescence Mounting Medium (Dako, Carpinteria, CA, USA) and imaged at 10× 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 ± S.E.M. or mean fold change ± 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 $\mu$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 $\mu$m $T_4$ resulted in significantly 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 tenasin-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 T3 and T4 via integrin αvβ3. MSCs were treated with HuH7 CM alone (grey line) or in the presence of 1 nM T3 or 1 μM T4 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) T3, (B) T4) 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).

Thyroid hormones T3 and T4 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 T3 or 1 μM T4 in the presence or absence of 100 nM tetrac before attaching to spheroids. Both after T3 (Fig. 4B) and T4 stimulation (Fig. 4D), MSCs showed deeper invasion (T3: 34.5 ± 7.42 μm; P = 0.15 and T4: 29.00 ± 1.37 μm; P < 0.01) into the centre of spheroids compared to untreated control cells (21.83 ± 1.79 μm; Fig. 4A and F). Cells treated with T3 or T4 in the presence of tetrac (T3 + tetrac: 9.00 ± 1.78 μm; P < 0.05 vs T3 and T4 + tetrac: 22.25 ± 1.55 μm; P < 0.01 vs T4) showed inhibition of invasion and remained at the surface (Fig. 4C, E and F). In some cases, especially after T3 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.

T4 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,
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\times10^5 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 ± 2.1%; P < 0.001; Fig. 5B) compared to euthyroid mice (CMFDA positive area 12.2 ± 1.0%; Fig. 5B). In hypothyroid mice, recruitment and invasion were strikingly reduced (CMFDA positive area 6.7 ± 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 ± 0.2%; P < 0.001; Fig. 5B). Neither thyroid hormone

Figure 3
Migration of MSCs towards tumour cell CM is increased upon T3 and T4 treatment mediated by integrin \( \alpha v \beta 3 \). (A) Representative trajectories of untreated MSCs without gradient influence. (B, C, D, E, F and G) Representative trajectories of MSCs treated with 1 nM T3 or 1 \( \mu \)M T4 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).
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 T3, T4 and tetrac. To distinguish between the effects on MSCs/stroma biology as opposed to direct effects on the tumour, we selected an αvβ3-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 T3 or T4, 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-β1, suggesting a T3/T4-enhanced CAF-like differentiation of MSCs mediated through αvβ3 as shown by inhibition of these effects by tetrac.

In line with our observations, transduction of the thyroid hormone signal via integrin αvβ3 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 (Herbergs et al. 2012). Integrin αvβ3 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, Herbergs 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 αvβ3 crosstalk. A further indirect effect could involve an enhanced expression of hypoxia-inducible factor 1 alpha (HIF-1α) that was shown to be increased upon T3 binding to integrin αvβ3 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 TGFBI (Davis et al. 2011, Herbergs et al. 2012). However, the exact mechanisms behind the synergistic effects of CM and T3/T4 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 αvβ3. Balzan et al. (2013) reported similar effects on microvascular endothelial cells in a wound-healing assay with increased migration after T3 or T4 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, αvβ3-mediated regulation of cell migration. Furthermore,
there is growing evidence that thyroid hormones increase the motility of αvβ3-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, Hercbergs 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 a$\nu$b3-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 T3 or T4 and/or T3 or T4 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 a$\nu$b3 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 T3 and T4 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 T3/T4 antagonistic action mediated through integrin a$\nu$b3 (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 a$\nu$b3 expressed on the tumour cells themselves. Using an a$\nu$b3-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 a$\nu$b3 expression on tumour cells.

In conclusion, our data suggest that thyroid hormones T3 and T4 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 a$\nu$b3. 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 T3 and T4 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.
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