BBA - General Subjects 1862 (2018) 1472–1481

Contents lists available at ScienceDirect

BBA - General Subjects

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

# Potential role for *Ext1*-dependent heparan sulfate in regulating *P311* gene expression in A549 carcinoma cells

Kirankumar Katta[1,2], Lawrence F. Sembajwe[2], Marion Kusche-Gullberg*

*Department of Biomedicine, University of Bergen, NO-5009 Bergen, Norway*

ARTICLE INFO

*Keywords:*
Heparan sulfate
EXT1
Fibroblasts
A549 adenocarcinoma cells
P311
TGF-beta

ABSTRACT

*Background:* Exostosin-1 (EXT1), a member of the EXT protein family, is indispensable for synthesis of heparan sulfate (HS) chains that bind to and modulate the signaling efficiency of numerous growth factor activities. We have previously shown that *Ext1* mutated mouse embryonic fibroblasts produce short sulfated HS chains which dramatically influence tumor cell behavior in a 3-dimensional (3D) heterospheroid system composed of tumor cells and fibroblasts.

*Methods:* In this study, we have used both 2D co-culture and 3D heterospheroid models, consisting of human A549 carcinoma cells co-cultured with wild-type or *Ext1*-mutated mouse embryonic fibroblasts.

*Results and conclusions:* Gene expression profiling of differentially expressed genes in fibroblast/A549 heterospheroids identified *P311* as a gene substantially down-regulated in A549 cells co-cultured with *Ext1*-mutated fibroblasts. In addition, we observed that the *Ext1* mutants displayed reduced *Tgf-β1* mRNA levels and lower levels of secreted active TGF-β protein. *Re*-introduction of *Ext1* in the *Ext1* mutant fibroblasts rescued the levels of *Tgf-β1* mRNA, increased the amounts of secreted active TGF-β in these cells, as well as *P311* mRNA levels in adjacent A549 cells. Accordingly, small interfering RNAs (siRNAs) against fibroblast *Tgf-β1* reduced *P311* expression in neighboring A549 tumor cells. Our data raises the possibility that fibroblast *Ext1* levels play a role in *P311* expression in A549/fibroblast co-culture through TGF-β1.

*General significance:* This study considers a possible novel mechanism of *Ext1*-regulated heparan sulfate structure in modifying tumor-stroma interactions through altering stromal *tgf-β1* expression.

## 1. Introduction

Exostosin (EXT)-1 and EXT2 are glycosyltransferases involved in heparan sulfate (HS) proteoglycan (PG) biosynthesis [1–3]. HSPGs, composed of one or more HS chains covalently attached to various protein cores, are ubiquitous components of the extracellular matrix (ECM) and play many important roles in tissue homeostasis [4,5]. They are essential for signal transduction of a multitude of signaling molecules thus driving processes such as cell survival, division, migration, differentiation and cancer development [5,6]. HSPGs are synthesized in a multistep process that involves chain elongation by the action of an EXT1/EXT2 *co*-polymerase complex. Concomitant with chain elongation, several modifications occur through an epimerase and various sulfotransferases that generate a complex polysaccharide containing *N*-acetylated and N-sulfated glucosamine residues, glucuronic acid and iduronic acid units, as well as O-sulfate groups in various positions [7]. Five genes encoding EXT proteins have been identified in mammals, *EXT1*, *EXT2*, and the *EXT*-like genes *EXTL1*, *EXTL2*, and *EXTL3*. *EXT1* and *EXT2* were first recognized as genes responsible for the autosomal inherited disorder hereditary multiple osteochondromas (also called hereditary multiple exostoses), by genetic linkage analysis [8–10]. The EXT-like proteins, EXTL1, EXTL2 and EXTL3, have not been linked to hereditary multiple osteochondromas. Instead, they were identified in screens for proteins homologous to EXT1 and EXT2 [11–13].

We have previously shown that *Ext1* mutated mouse embryonic fibroblasts ($Ext1^{Gt/Gt}$) produce short sulfated HS chains with relatively intact sulfation pattern [14]. The average molecular sizes of the HS chains from wild-type ($Ext1^{wt/wt}$) and mutated ($Ext1^{Gt/Gt}$) mouse embryonic fibroblasts were estimated to be 70 and 12.5 kDa, respectively. The mutated fibroblasts show a reduced proliferation rate, a reduced FGF2 signaling response and a reduced ability to adhere to and to remodel collagen gels [15]. We further used a three-dimensional (3D) mini-tumor spheroid model of human tumor cell lines and mouse fibroblasts to study the cross-talk between these two major and mutually

* Corresponding author.
*E-mail address:* marion.kusche@uib.no (M. Kusche-Gullberg).
[1] Current address: Center for Eye Research, Department of Ophthalmology, Oslo University Hospital Ullevål, Oslo, Norway.
[2] Kirankumar Katta and Lawrence F. Sembajwe contributed equally to this work.

https://doi.org/10.1016/j.bbagen.2018.03.024
Received 7 November 2017; Received in revised form 14 February 2018; Accepted 21 March 2018
Available online 24 March 2018
0304-4165/ 

dependent components by providing a microenvironment more similar to a primary tumor. In mixed multicellular 3D-spheroids, composed of human tumor cells and *Ext1*$^{wt/wt}$ or *Ext1*$^{Gt/Gt}$ fibroblasts, the *Ext1* mutation in stromal fibroblasts strongly influences tumor cell behavior and the interstitial fluid pressure [16]. *Ext1*$^{Gt/Gt}$/A549 lung adenocarcinoma cell heterospheroids have a homogenous low interstitial fluid pressure throughout the spheroid, whereas the corresponding data for *Ext1*$^{wt/wt}$/A549 spheroids vary greatly with the depth of measurements. After 6 days in culture, the wild-type fibroblasts form an inner core and the tumor cells an outer layer of cells. For spheroids containing *Ext1*$^{Gt/Gt}$ fibroblasts, this segregation is less obvious, indicating impaired tumor cell migration that coincides with a lower proliferation rate. The *Ext1*-dependent migration behavior was not restricted to A549 cells, as similar cell behaviors were observed also with H460- and HeLa cell-containing heterospheroids [16].

To further investigate the role of *Ext1* expressed by stromal fibroblasts in modulating the lung adenocarcinoma cells in *Ext1*$^{Gt/Gt}$/tumor heterospheroids we in this study screened for differently expressed human genes in human A549 tumor cells interacting with *Ext1*$^{Gt/Gt}$ fibroblasts. Using microarray analysis we identified the *P311* (*C5ORF13*) gene as downregulated in *Ext1*$^{Gt/Gt}$/A549 heterospheroids. *P311* encodes the P311 protein, also known as NREP (Neuronal Regeneration Related Protein) or PTZ17 (pentylenetetrazol-17) [17]. P311 is a PEST [rich in proline (P), glutamic acid (E), serine (S), and threonine (T)]-domain containing 8-kDa intracellular protein with a short half-life of approximately 5 min [18]. It was originally found in developing neurons and muscles but is expressed ubiquitously in several other tissues as e.g. muscle, lung, regenerating tissues [19] and invasive glioma cells [20]. We further show that the mRNA expression of *P311* in A549 tumor cells is regulated by the cytokine transforming growth factor beta-1 (TGF-β1).

## 2. Material and methods

### 2.1. Cell culture

No experiments on live vertebrates were performed; the cells/cell lines described below were either purchased or given to us as a gift and have been used in our lab for > 12 years. Mouse embryonic fibroblasts derived from wild-type and *Ext1* gene trapped *Ext1*$^{Gt/Gt}$ (official designation, *Ext1*$^{Gt(pGT2TMpfs)064Wcs}$, [21]) (kindly given by Dr. Andrea Vortkamp, University of Duisburg-Essen, Essen, Germany) were Sv40-immortalized as described in [14]. A549, a human non-small cell lung adenocarcinoma cell line, was purchased from the American Type Culture Collection (ATCC). Mink lung epithelial cells (TMLC) were kindly provided by Dr. Dean Sheppard (UCSF Medical Center, CA, USA). Monolayer cell cultures and multicellular heterospheroids were cultured in Dulbecco's modified Eagles medium (DMEM) with Glutamax (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 units/ml of penicillin and 0.1 mg/ml of streptomycin (Sigma) and cultured at 37 °C and 5% $CO_2$.

### 2.2. Construction of Ext1 expression plasmid and transfection of HEK 293 cells

For reintroduction of *Ext1* into *Ext1*$^{Gt/Gt}$ fibroblasts, a full-length mouse *Ext1* cDNA was excised from pBudCE4.1 vector [22] using *Eco*RI restriction sites and subcloned into the corresponding site of retroviral pBABE plasmid vector (Addgene) carrying the puromycin resistant gene (pBabe puro IRES-EGFP). Enzyme restriction digestions confirmed the orientation of the insert with *Ext1* in frame with the C-terminal EGFP tag. For stable expression, the vector carrying the *Ext1* cDNA or the empty vector (mock) were transfected into retroviral packaging Phoenix cells (HEK293T cells constructs capable of producing gag-pol, and envelope protein for viruses). Subsequently, the culture medium from the transfected Phoenix cells containing the retrovirus particles was collected to infect the *Ext1*$^{Gt/Gt}$ cells. After infection, cells were cultured in the presence of 2 μg/ml of puromycin (Sigma). Three cellular populations (referred to as cl.1, cl.2 and cl.3) stably expressing *Ext1* at relatively low, medium and high levels were isolated by flow cytometry based on the level of EGFP expression.

### 2.3. Gene silencing with siRNA

Pre-designed siRNAs for mouse TGF-β1 were from Ambion and non-targeting control siRNA was from Dharmacon. Sequences of primers are listed in Supplementary Table S1. Wild-type *Ext1* (*Ext1*$^{wt/wt}$) cells were transfected with the siRNA (50 nM of each) using lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). After 48 h cells were trypsinized and co-cultured with A549 cells in 1:1 ratio for an additional 24 h. mRNA levels were evaluated by real time PCR using human and mouse specific primers as described below.

### 2.4. 2D co-culture experiments

*Ext1*$^{wt/wt}$ or *Ext1*$^{Gt/Gt}$ cells and A549 cells were seeded at 1:1 ratios in a 6-well plate (125,000 cells of each cell type per well). After co-culturing for 48 h mRNA levels were evaluated by real time PCR using human and mouse specific primers (listed in Supplementary Table S1) as described below.

### 2.5. Preparation and culturing of spheroids

Composite spheroids were prepared by co-culturing *Ext1*$^{wt/wt}$ or *Ext1*$^{Gt/Gt}$ mouse embryonic fibroblasts with A549 tumor cells using the hanging drop method [23,24] as described in [16]. Briefly, sub-confluent cells were trypsinized and suspended in culture medium to a concentration of $1 \times 10^6$ cells/ml. Single cell suspension of fibroblasts and tumor cells were mixed in a ratio of 9:1 and 25 μl of the composite cell suspension ($2.5 \times 10^4$ cells) were pipetted onto the lid of a cell culture dish to form one drop. The lid was then inverted and placed over a cell culture dish containing DMEM for humidity and cultured under standard conditions for 6 days.

### 2.6. Microarray analysis in composite spheroids

Human specific microarray analysis was performed on 6-day old composite spheroids. Five different replicates were made for each spheroid type. For RNA preparation, the spheroids were grinded in RLT buffer using a mixer mill homogenizer (Retsch, Germany). Total RNA was prepared from the homogenized spheroids using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Extracted total RNA was quality tested on Agilent Bioanalyzer 2100 and a human specific microarray was performed at the NMC-UoB Microarray Core Facility using the Illumina Bead Array Technology (HumanHT-12 v4 Expression Bead Chip). The raw microarray data was quality examined in GenomeStudio and SampleProbeProfile-text file was exported from GenomeStudio, during which, control probes were removed. The resulting gene expression table was imported into J-Express 2012 (http://jexpress.bioinfo.no/site/) for further quality control and analysis. Differentially expressed human genes between the two groups of samples (RNAs from *Ext1*$^{wt/wt}$/A549 and *Ext1*$^{Gt/Gt}$/A549) were analyzed using SAM method and only the genes with q-value < 10 were considered to be valid. The datasets generated and analyzed during the current study are available in the ArrayExpress repository, ID: E-MTAB-5874.

### 2.7. Quantitative real time PCR (RT-PCR)

Total RNA was isolated from cells in monolayer cultures and heterospheroids using RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. Aliquots of 1 μg of total RNA were reverse transcribed