Experimental Cell Research 339 (2015) 35–43

Contents lists available at ScienceDirect

Experimental Cell Research

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

Research Article

A novel 3D high-content assay identifies compounds that prevent fibroblast invasion into tissue surrogates

Carsten Wenzel, Saskia Otto, Stefan Prechtl, Karsten Parczyk, Patrick Steigemann *

Bayer Pharma AG, Global Drug Discovery, Berlin, Germany

ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form
29 September 2015
Accepted 2 October 2015
Available online 21 October 2015

Keywords:
3D co-culture
High-content invasion assay
Living tissue surrogate
Fibroblast invasion
Prostaglandin
ROCK

ABSTRACT

Invasion processes underlie or accompany several pathological processes but only a limited number of high-throughput capable phenotypic models exist to test anti-invasive compounds *in vitro*. We here evaluated 3D co-cultures as a high-content phenotypic screening system for fibrotic invasive processes. 3D multicellular spheroids were used as living tissue surrogates in co-culture with fluorescently labeled lung fibroblasts to monitor invasion processes by automated microscopy. This setup was used to screen a compound library containing 480 known bioactive substances. Identified hits prevented fibroblast invasion and could be subdivided into two hit classes. First, Prostaglandins were shown to prevent fibroblast invasion, most likely mediated by the prostaglandin EP_2 receptor and generation of cAMP. Additionally, Rho-associated protein kinase (ROCK) inhibitors prevented fibroblast invasion, possibly by inactivation of myosin II. Importantly, both Prostaglandins and ROCK inhibitors are potential treatment options shown to be effective in *in vitro* and *in vivo* models of fibrotic diseases. This validates the presented novel phenotypic screening approach for the evaluation of potential inhibitors and the identification of novel compounds with activity in diseases that are associated with fibroblast invasion.

1. Introduction

Uncontrolled fibroblast recruitment, migration and invasion is prevalent in different kind of malignancies [1]. In certain types of cancer so called cancer associated fibroblasts (CAFs) are recruited by neoplastic cells to promote tumor growth, survival, and progression [2]. CAFs can constitute large parts of the tumor and can promote local cell invasion and metastasis and could comprise an important target for cancer therapy e.g. by interference with CAF recruitment or invasion [1–6]. In idiopathic pulmonary fibrosis (IPF) aberrant migration and proliferation of fibroblasts into normal lung tissue leads to a decrease of oxygen capacity and finally to death in IPF patients [1,7] and the accumulation of ECM-producing myofibroblasts is thought to be one of the key features of lung fibrosis pathophysiology [1,7,8].

Accordingly reduction of fibroblast invasion is discussed as treatment strategy in a variety of diseases, *e.g.* to protect against IPF by preventing lung tissue destruction [9–11] or to slow tumor progression and invasion by targeting CAFs [1–4,6].

Despite fibroblast invasion possibly being an integral feature of different malignancies, little is known about the cellular and molecular mechanisms that underlie fibroblast recruitment and fibrotic tissue invasion. The lack of in-depth understanding of the molecular mechanisms underlying these processes exacerbates the development of novel potential treatment options based on targeted approaches. In contrast, phenotypic screening approaches do not require in-depth understanding of the molecular mechanisms of a disease [12] and therefore represent an attractive option for the identification and development of novel treatment regimens.

2D cell culture settings lack complex ECM composition, stiffness and cell/cell or cell/matrix interactions, all being crucial components of cellular invasion into tissue. Therefore, due to the complex pathophysiology, only few cell based phenotypic *in vitro* assays that focus on fibroblast invasion have been reported [6,9,10,13–15], all relying on the use of artificial and acellular matrices and most of them not being screening-compatible.

In the last years, 3D cell culture models are increasingly utilized in drug discovery and have the potential to mimic the complex three dimensional organization of tissue *in vivo* [16–18]. Indeed, an increasing amount of studies make use of complex, heterogeneous 3D co-culture models to mimic interaction, migration and invasion processes *in vitro* [17,19–24].

The here presented novel 3D co-culture assay for tissue invasion uses for the first time 3D multicellular spheroids to provide a living tissue-like surrogate composed of cells in combination with

Abbreviations: IPF, idiopathic pulmonary fibrosis; MTCS, multicellular spheroid; ROCK, Rho-associated protein kinase; ECM, extracellular matrix; CAF, cancer associated fibroblasts

* Correspondence to: Bayer Pharma AG, Global Drug Discovery, Muellerstrasse 178, 13353 Berlin, Germany.
E-mail address: Patrick.Steigemann@bayer.com (P. Steigemann).

http://dx.doi.org/10.1016/j.yexcr.2015.10.003
0014-4827/

fibroblasts to mimic fibrotic invasion processes into living tissue. Automated fluorescent imaging techniques allow spatial and temporal monitoring of invasion and migration of lung fibroblasts. Moreover, subsequent automated image analysis enables compound characterization of fibrotic invasive processes into living tissue.

By High-Content Screening we identified 16 substances from a commercial drug library that inhibit invasion of fibroblasts into spheroids. By further experiments the hits could be subdivided into two hit classes, Prostaglandin EP_2 agonists and Rho-kinase inhibitors. Interestingly, both hit classes have been reported to show activity in preclinical models of diseases that are associated with fibroblast invasion, validating the presented approach for the phenotype-based identification of compounds with potential activity against fibrotic diseases [25–28]. In conclusion, the presented novel phenotypic co-culture screening assay setup could aid the evaluation of compounds for the treatment of diseases that involve fibroblast tissue invasion and identification of novel potential treatment options for invasive fibrotic processes.

2. Materials and methods

2.1. Spheroid generation

Spheroid generation was carried out using a modified version of the liquid overlay cultivation technique described previously [16,18]. For the generation of imaging-compatible 3D spheroid fibroblast co-cultures, 10 μl of a heated 1.5% (w/v) agarose (in DMEM without phenol red) solution was dispended by a liquid dispenser (Multidrop Combi, Thermo Scientific) into sterile 384-well clear bottom imaging plates. To prevent premature gelation of the agarose suspension, the Multidrop and dispensing cassette was heated by infrared lamps. For spheroid seeding, a single-cell suspension was seeded into agarose-coated (1.5% (w/v)) 384-well clear bottom plates in 40 μl RPMI1640 containing 10% (v/v) FBS supplemented with 1% (v/v) Penicillin/Streptomycin (and 0.01 μg/ml Insulin for T47D cells (Gibco)) using a liquid dispenser. The plates containing single cell suspensions on agarose were incubated under standard cell culture conditions at 37 °C and 5% CO_2 in humidified incubators for 4 days to allow formation of reproducible spheroids of defined size and morphology [18]. To obtain spheroids with an approximate diameter of 400 μm on day 4, 2000 cells were seeded per well for the breast cancer cell line T47D, 1000 cells for the breast cancer cell line MCF7 and 1000 cells for the lung cancer cell line H1299.

2.2. Fibroblast staining

After spheroid formation, fluorescently labeled fibroblasts were added to the spheroid containing wells. For this, non-toxic live cell stain Vybrant DiD (Vybrant DiD Cell-Labeling Solution, Life Technologies) was used to stain fibroblasts for visualization of invasion according to the protocol. Cells were collected in 2–4 mL serum-free media ($\sim 1 \times 10^6$/ml) and incubated with Vybrant DiD (f.c. 1:200) for 15 min at 37 °C. Cells were washed once with 10 mL of FCS-containing media, centrifuged (5 min, 800 rpm) and re-suspended in 5 mL warm culture medium for cell counting and further use. Fibroblast cell lines were obtained from ATCC, Human Pulmonary Fibroblasts (HPF) from PromoCell. 2000 Fibroblasts cells were then added in 20 μl to each spheroid-containing well in the 384 W microtiter plate.

2.3. Compound treatment

Eighty nanoliter compounds (ENZO Screen-Well ICCB Known Bioactives library (480 compounds)) were added in 20 μl culture medium simultaneously with 20 μl of the invading cell type MRC-5 (2000 c/w) for additional 3 days ending up in a final volume of 80 μl per well and a final compound dilution of 0.1–20 μM, depending on original stock concentration. As controls DMSO (solvent control, 1:1000), Antimycin A (respiratory chain inhibitor, growth arrest control at 100 nM), Cytochalasin D (Actin cytoskeleton disruptor, general migratory inhibitor control at 4 μM) and Staurosporine (general toxic control at 10 μM) were used.

Receptor agonists and antagonists (All Prostaglandins (E1, E2, F2, A1, A2, J2), L798106, SC51322, PF04418948, U46619, Sulprostone, Cloprostenol, Butaprost, Fluprostenol, and Misoprostol) were purchased from Tocris Bioscience. Forskolin (cAMP activator), Blebbistatin, H7, H71007 and Y-27632 (ROCK inhibitor) were purchased from Sigma-Aldrich. Rho-kinase inhibitor Azaindole-1 was provided from the Bayer Stock Library. All compounds were dissolved in DMSO (10 mM) and stored at −20 °C.

2.4. Spheroid staining

Prior to imaging, spheroids were stained for over night by adding Hoechst 33342 (1 mg/ml, Life Technologies) as counterstain for all nuclei and Sytox Green, as stain for dead cells (2 mM, Life Technologies) at a final dilution of 1:10000 each. For additional experiments the outer spheroid cell layer was stained with CalceinAM (Thermo Fischer Scientific, C1430).

2.5. Image acquisition and analysis

One image per spheroid and wavelength, focused on the spheroid center were captured by an Opera confocal spinning disc microscope system with a 4× objective (4× Air Objective, Nikon Japan, Plan Apo, 4×/0.2, ∞/-WD 15.7). Total spheroids were stained with Hoechst, dead cells stained with Sytox Green and the fibroblasts labeled with Vibrant DiD. Quantification of cell death and fibroblast invasion area was done with MetaXpress software (Molecular Devices) using custom written image analysis routines. To identify and exclude general toxic compounds spheroid borders were detected on the Hoechst channel and masks were generated, scaled down (spheroid mask) and transferred to the Sytox Green channel, in which cell death was quantified by intensity measurement of the Sytox Green signal. For evaluation of fibroblast invasion, the spheroid mask was transferred to the fibroblast channel, and the area of the invaded fibroblasts was quantified by measurement of the area of Vibrant DiD staining above a fixed background value and normalized to the total spheroid area. All images were captured as 12-bit tiff files and no non-linear corrections have been applied.

Normalization, quality control and fitting curves for AC_{50} determination of identified hit compounds were done with Genedata Screener® for high-content screening and Genedata Condoseo modules (Genedata AG). In detail, wells with no recognizable spheroid were masked. Subsequently, the intensity measurement of the dead cell signal (Sytox Green) was normalized to DMSO (0%) and 10 μM Staurosporine (100%) controls and compounds with >30% signal excluded from analysis as inducers of general toxicity. The relative area covered by fibroblasts (area measurement of fibroblasts divided by total spheroid area) was normalized to DMSO (0) and 1 μM PGE2 (−100) and curves were fitted to S0=0 and Sinf=−100. The relative spheroid area covered by the fibroblast signal was 0.433 ± 0.01 ($n=128$) for the DMSO control (CV=2,3%), in contrast to 0.224 ± 0.006 for the treatment control (1 μM PGE2; $n=128$) (CV=2.5%), giving a S/B ratio of 1.94 and a negative (robust) *Z*-factor, due to high variability in the control wells.