International Journal of Biochemistry and Cell Biology 88 (2017) 145–154

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

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

Lung inflammation after bleomycin treatment in mice: Selection of an accurate normalization strategy for gene expression analysis in an *ex-vivo* and *in-vitro* model

Veronica Della Latta[a,1], Manuela Cabiati[a,1], Silvia Burchielli[b], Giada Frenzilli[c], Margherita Bernardeschi[c], Antonella Cecchettini[c], Federica Viglione[a], Maria-Aurora Morales[a], Silvia Del Ry[a,*]

[a] *CNR Institute of Clinical Physiology, Laboratory of Biochemistry and Molecular Biology, Pisa, Italy*
[b] *Fondazione Gabriele Monasterio, Pisa, Italy*
[c] *University of Pisa, Dept. Experimental and Clinical Medicine, Pisa, Italy*

ARTICLE INFO

Keywords:
Pulmonary fibrosis
Bleomycin
Reference genes
Real-Time PCR
PTX-3
TNF-α

ABSTRACT

Pulmonary fibrosis (PF) is the most common and aggressive interstitial lung disease, characterized by a patchy development of fibrosis leading to progressive destruction of the normal lung architecture which is preceded by an inflammatory process. Gene expression studies are important to understand the development of PF but the accuracy and reproducibility of Real-Time PCR depend on appropriate normalization strategies. This study aimed to analyze the expression variability of eight commonly used reference genes during the initial inflammatory phase of bleomycin-induced PF in a mouse model and to verify whether the selected reference genes could be applied to an *in-vitro* model of BLM-treated primary murine lung fibroblasts. Wild-type C57BL/6 mice (n = 40) were used. Real-Time PCR was carried out on lung tissue of mice either BLM (BLM-tm) or physiological solution-treated (PSS-tm), and in primary lung fibroblasts, isolated from healthy C57BL/6 mice. Histological analysis was performed to confirm the inflammation development. During inflammation, the most stable genes resulted: PPIA, HPRT-1 and SDHA for both models; the normalization strategy was tested analyzing mRNA expression of PTX-3 and TNF-α which resulted up-regulated both in *ex-vivo* and *in-vitro* with respect to PSS-tm/fibroblasts. Histological analysis supported the results. This study identified a new set of reference genes expressed both in the *in-vitro* and *ex-vivo* models. A higher expression of both markers in BLM-tm with respect to PSS-tm indicated that BLM might lead to increased PTX-3 local production by a co-regulation with TNF-α at lung level.

1. Introduction

Interstitial lung diseases (ILDs) are a heterogeneous non-neoplastic group of more than 200 different diseases with variable etiology, such as autoimmunity, medications, radiation or exposure to substances (*e.g.* asbestos, coal, silica). They are characterized by different degrees of fibrosis and inflammation leading to progressive destruction of the normal lung architecture (Demedts et al., 2001), since the interstitium, the airspaces, peripheral airways, and vessels along with their respective epithelial and endothelial linings are affected (American Thoracic Society, 2001). In this context, idiopathic pulmonary fibrosis is the most aggressive interstitial lung disease associated with the histological appearance of usual interstitial pneumonia on lung biopsy. Pulmonary fibrosis (PF) is characterized by cellular proliferation and progressive accumulation of extracellular matrix constituents resulting in remodeling of the lung interstitium (Selman et al., 2001; Thannickal et al., 2004; Gross and Hunninghake, 2001; Raghu et al., 2011).

To date, the molecular mechanisms and potential genetic pathways responsible for PF development have not been yet identified, although inflammation seems to be one of the leading causes of disease initiation and progression (Selman and Pardo, 2002; Scotton and Chambers, 2007; Della Latta et al., 2015).

The murine model represents the most widely used animal model to study the fibrotic process. (Moeller et al., 2006; Moore and Hogaboam, 2008; Degryse and Lawson, 2011). Different approaches have been used to induce PF. Bleomycin (BLM), an anti-neoplastic drug, is commonly

* Corresponding author at: CNR Institute of Clinical Physiology, Via Giuseppe Moruzzi 1, 56124, Pisa, Italy.
E-mail address: delry@ifc.cnr.it (S. Del Ry).
[1] Contributed equally to this work.

http://dx.doi.org/10.1016/j.biocel.2017.05.016
Received 14 September 2016; Received in revised form 10 April 2017; Accepted 8 May 2017
Available online 16 May 2017

used to induce lung fibrosis in the experimental setting (Umezawa et al., 1966; Grande et al., 1998; Walters and Kleeberger, 2008). Intratracheal administration of BLM is able to stimulate lung injury inducing an inflammatory process within a week after administration and resulting then in fibrosis around days 21–28 (Moore and Hogaboam, 2008; Peng et al., 2013; Della Latta et al., 2013; Robbe et al., 2015). The inflammatory process starts 2 days after BLM-instillation and its development is well highlighted after 7 days both by histological and bio-humoral evaluations. Subsequently, the onset of fibrosis can be also observed by day 14, with a maximal response usually recorded after day 21. In the murine model, lung impairment after intratracheal BLM is self-limiting, since the fibrosis resolves after 28 days of drug administration. Lung histology in BLM-treated animals shows a highly heterogeneous and patchy distribution of lung fibrosis, honeycombing, fibroblastic foci and a paucity of inflammation, similar to what observed in human PF (Selman et al., 2001).

Gene expression studies are an integral part to understand the development of PF and Real-Time PCR is the benchmark method for molecular analysis of biological systems, being a rapid, highly reproducible and extremely sensitive technique (Dabek et al., 2010; Veys et al., 2012). However, the reliability of Real-Time PCR results suffers from experimental conditions, as yield and quality of the sample RNA, the efficiency of reverse transcription and primer design (Fleige and Pfaffl, 2006). Additionally, several studies reported how the accuracy and reproducibility of Real-Time PCR data are closely dependent on appropriate normalization strategies to reduce the background noise of the method (Bustin, 2000; Vandesompele et al., 2002; Huggett et al., 2005; Hendriks-Balk et al., 2007; Martino et al., 2011). Thus, specific reference genes are selected for calibration and normalization of target biomarkers in order to reduce the standard deviation of experimental data (Pierzchala et al., 2011). The expression levels of frequently used reference genes show a significant variation across cell types, tissues, organs, metabolic conditions, experimental treatments as well as *in-vitro* cellular cultures (Chechi et al., 2012). To date, no indication is available in the potential translation of gene expression results from *in/ex-vivo* model of lung disease, as PF, to *in-vitro* cellular model. The aim of this study was to analyze the expression variability of eight commonly used reference genes during the initial inflammatory phase of BLM-induced PF in lung tissue of a mouse model and to verify whether the selected reference genes could be applied to an *in-vitro* model of BLM-treated primary murine lung fibroblasts. The inflammatory process, which is clearly evident after around a week of BLM instillation, seems to involve several acute phase reactants; among them a classical marker, as the tumor necrosis factor (TNF)-α and the novel vascular inflammatory marker Pentraxin (PTX)-3 play a role in regulating immune system in several pulmonary pathologies. Thus, the set of suitable reference genes selected was used to evaluate the possible expression profile variation of TNF-α and PTX-3 which are involved in the inflammation phase before PF development.

2. Materials and methods

2.1. Ethics statement

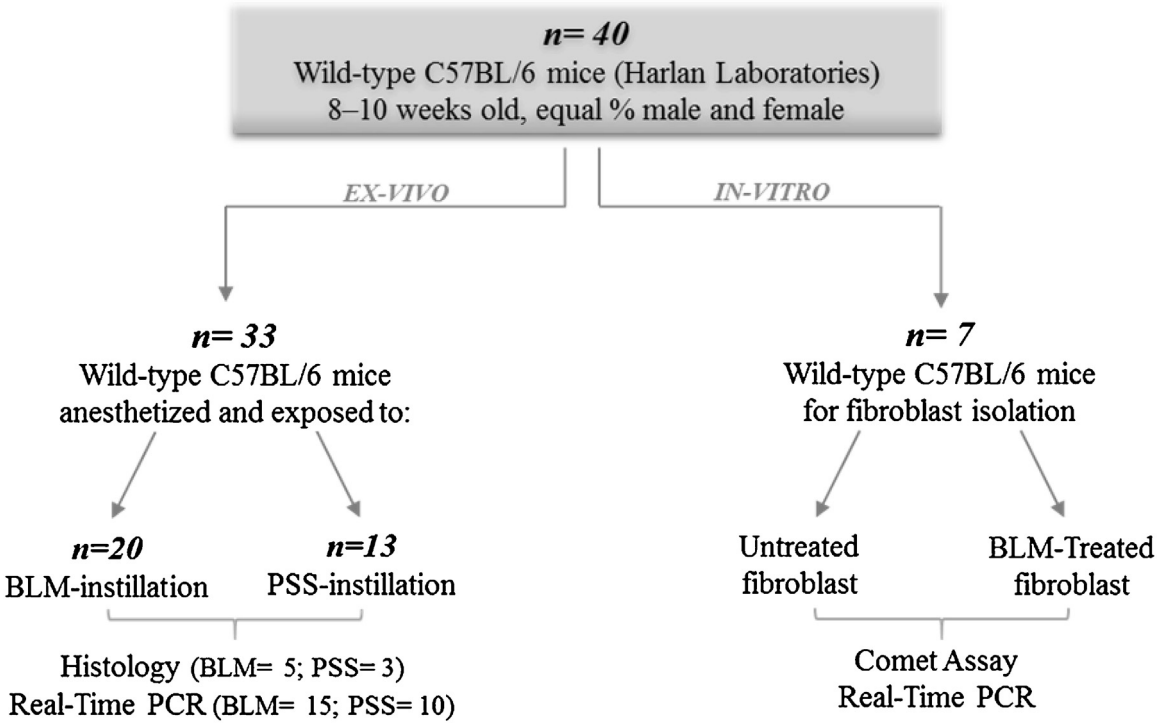
National guidelines for the care and use of research animals (D.L. 116/92, implementation of EEC directive 609/86) were followed.

2.2. Experimental protocols

A total of 40 wild-type C57BL/6 mice (Harlan Laboratories) 8–10 weeks old, female and male in equal percentage were used to build both *ex-vivo* and *in-vitro* settings (Scheme 1).

2.2.1. Ex-vivo murine model of PF induction: inflammatory process monitoring

Out of 40 mice, a first group (n = 33), anesthetized with Ketamine/xylazine (Virbac, srl) ketamine 80 mg/kg intraperitoneal; xylazine 10 mg/kg intraperitoneal, was exposed in part (n = 20) to BLM (3U/kg of drug dissolved in 0.1 ml saline solution, Sigma-Aldrich, Milan, Italy) by intratracheal instillation (BLM group, weight = 26.6 ± 0.4 g), while the remaining littermates (n = 13) were exposed to intratracheal instillation of physiological saline solution (PSS group, 26.7 ± 0.5 g). All mice were monitored and sacrificed after 7 days of treatment. Each procedure was performed under anesthesia, and all



Scheme 1. Experimental protocol related to wild-type C57BL/6 mice groups used in ex-vivo and in-vitro settings. (BLM = bleomycin; PSS = physiological saline solution).