

Development of Immortalized Human Cell Lines from TERT-Transfected Multi-lineage Progenitor Cells (MLPC) by Differentiation or Cell Fusion: Models for SARS-CoV-2 Binding and Infectivity

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Abstract

MLPC are human cord blood-derived non-hematopoietic stem cells with extensive proliferative and differentiation capacity. TERT transfection of MLPC rendered them functionally immortal. Methods to create immortalized fully mature cells by differentiation or by direct fusion of TERT-MLPC to primary cells are described. Hepatocyte-like cells (HLC) created by differentiation and fusion displayed markers and functionality consistent with well-differentiated human hepatocytes. Differentiation of MLPC into alveolar type 2-like (AT2) cells displayed markers consistent with human primary small airway epithelial cells. Potential interactions of the SARS-CoV-2 with both cell lines were investigated by binding of spike proteins and their inhibition by spike-specific and marker-specific antibodies. Spike and spike 1 binding with AT2 cells occurred via surface ACE-2 receptors. Spike binding to both HLC and primary human hepatocytes (PHH) was mediated by interactions with the hepatocyte surface membrane asialoglycoprotein receptor 1 (ASG1).

Keywords: Multi-lineage Progenitor Cells; Hepatocyte-like cells; AT2-like cells; Hepatocyte fusion cells; SARS-CoV-2; Spike proteins

Description

In vitro studies using human primary cells are hindered by limited viability, inability to proliferate *in vitro*, donor-to-donor functional variability, availability and expense. The intent of these studies was to produce long-lived and proliferative cell lines with the characteristics of mature human primary cells as an *in vitro* cellular platform for functionality and viral interaction assays.

Multi-lineage progenitor cells (MLPC) were isolated from human post-partum umbilical cord blood. MLPC differ from other mesenchymal-like cells by gene expression, markers associated with stemness (OCT-4 and SOX-2), proliferative capacity (up to 80 population doublings) and the ability to be differentiated to non-mesodermal outcomes [1-3]. Transfection of the MLPC with the gene for telomerase reverse transcriptase (TERT) resulted in functionally immortalized cells that retained differentiation capacity. Single cell cloning resulted in the establishment of a cell line, E12, with the greatest capacity for differentiation. This cell line has been grown for >14 years while maintaining chromosomal stability and differentiation capacity and was used throughout these studies [4,5].

Immortalized cell lines with the characteristics and functionality of primary human hepatocytes were created by two methods. In one, E12 cells were differentiated to hepatocyte-like cells in a three-step procedure. E12 cells were differentiated to committed endodermal cells by a 5-7 day culture in medium supplemented with 100 ng/ml activin A. Cells were then further differentiated to committed hepatocyte precursors by culture for 14 days in medium supplemented with FGF basic 1 (20 ng/ml), FGF-4 (20 ng/ml), HGF (40 ng/ml), SCF (40 ng/ml), Oncostatin M (20 ng/ml), BMP-4 (20 ng/ml), EGF (40 ng/ml) and IL-1 beta (20 ng/ml). Final differentiation to fully mature hepatocyte-like cells (HLC) required an additional 7 day culture in medium supplemented with FGF basic (20 ng/ml), FGF-4 (20 ng/ml), HGF (40 ng/ml), SCF (40 ng/ml), Oncostatin M (20 ng/ml), BMP-4 (20 ng/ml), EGF (40 ng/ml), IL-1 beta (20 ng/ml), 0.5% DMSO and retinoic acid (30 µg/ml) [4]. In a second methodology, E12 cells were directly fused to primary human hepatocytes by PEG [5].

Differentiation of E12 cells to HLC was characterized by sequential expression of markers consistent with committed endodermal cells (SOX-17 and GATA-4), hepatocyte precursor cells (α -fetoprotein, albumin) and final differentiation to mature hepatocytes (translocation of hepatocyte nuclear factor-4 to the nucleus). Fusion of E12 and PHH resulted in full attainment of well-differentiated hepatocyte markers without further processing. Functional studies demonstrated that both methods resulted in cells that produced both urea and albumin.

Immortalized AT2-like cells were created by a single-step differentiation procedure, using an 8-10 day culture in small cell airway growth medium (SAGM, Lonza, Walkersville, MD). Resultant cells expressed markers consistent with AT2 cells (HT2-280, TM4SF1, surfactant protein C and ACE-2) without expression of AT1 markers (AGER, aquaporin and caveolin-1). Subsequent culture of the AT2-like cells demonstrated that they did not further progress to AT1 cells. ACE-2 has been demonstrated to be a key binding site for the SARS-CoV-2 virus [6-10]. Strong expression of ACE-2 on both E12 AT2 and primary SA-Epi cells suggested these cells could be useful tools to investigate SARS-CoV-2 interactions via their affinity for spike protein binding to ACE-2 receptors [11].

Potential interactions of the SARS-CoV-2 with E12 AT2 cells, SAEpi cells, E12 fusion cells and primary human hepatocytes were studied using biotinylated spike and spike 1 proteins and streptavidin-alexa 594 to visualize the binding of the spike proteins by confocal analysis [11,12]. Specificity of spike binding was confirmed by blockage

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Received: February 17, 2021; **Accepted:** March 03, 2021; **Published:** March 10, 2021

Citation: Collins DP, Steer CJ (2021) Development of Immortalized Human Cell Lines from TERT-Transfected Multi-lineage Progenitor Cells (MLPC) by Differentiation or Cell Fusion: Models for SARS-CoV-2 Binding and Infectivity. J Cytokine Biol 6: 38.

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of biotinylated spike proteins by unlabeled spike protein. E12 AT2 cells and SAEpi cells were capable of binding both spike (receptor binding domain positive, RBD) and spike 1 proteins (also containing the RBD). Subsequent to binding, spike protein was internalized on both E12 AT2 and SAEpi cells [11]. Fusion hepatocyte-like cells and primary hepatocytes were negative for the ACE-2 receptor. Yet they were able to bind intact spike protein, but not spike 1, suggesting a binding mechanism to hepatocytes independent of the RBD [12]. Two commercially available neutralizing antibodies were tested for their ability to block the binding of biotinylated spike proteins. One antibody was able to block the binding of spike protein to the E12 AT2 cells and SAEpi cells while the other was not able to block binding. When tested with the fusion HLC and PHH, both antibodies were able to inhibit binding. Taken together these results suggested that some neutralizing antibodies may function independently of spike binding to the ACE-2 receptor. Inhibition of spike binding to HLC and PHH by an antibody directed to the asialoglycoprotein receptor 1 strongly suggests that ASGr1 may act as a mechanism for the SARS-CoV-2 virus to bind and infect hepatocytes *via* clathrin-mediated endocytosis [12,13].

Immortalized E12 AT2 cells and fusion HLC provide a stable, reproducible source of cells to study the interactions of SARS-CoV-2 with a multitude of cell surface receptors. Furthermore, they could provide a valuable tool in the development of therapeutics designed to inhibit viral binding, fusion of virus membrane and host cell membrane, and inhibition of binding to non-ACE-2 sites on host cells. With the emergence of new variants of SARS-CoV-2, it is critical to understand the relationship between viral binding, infectivity and pathology. These cells can provide a novel and reproducible platform to study those interactions.

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