Cytometric analysis, genetic manipulation, and antibiotic selection of the snail embryonic cell line Bge from *Biomphalaria glabrata* the intermediate host of *Schistosoma mansoni*

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Abstract

The invertebrate cell line, Bge, from embryo of the snail *Biomphalaria glabrata* remains to date the only established cell line from any species of the Phylum Mollusca. Since its establishment in 1976 by Eder Hansen, few studies have focused on profiling its cytometrics, growth characteristics or sensitivity to xenobiotics. Bge cells are reputed to be challenging to propagate and maintain. Therefore, even though this cell line is a noteworthy resource, it has not been studied widely. With growing interest in functional genomics, including genetic transfection, to elucidate molecular aspects of the snail intermediate hosts responsible for transmission of schistosomiasis, and aiming to enhance the convenience of maintenance of this molluscan cell line we deployed the xCELLigene real time approach to study Bge cells. Doubling times for three isolates of Bge, termed CB, SL and UK, were longer than for mammalian cell lines - longer than 40 hours in complete Bge medium supplemented with 7% fetal bovine serum at 25^{o}C , ranging from ~42 hours to ~157 hours when 40,000 cells were seeded. To assess the potential of the cells for genetic transformation, antibiotic selection was explored. Bge cells were sensitive to the aminonucleoside antibiotic puromycin (from *Streptomyces alboniger)* from 5 µg/ml to 200 ng/ml, displaying a half maximal inhibitory concentration (IC50) of ~1.91 µg/ml. Sensitivity to puromycin, and relatively quick kill time (<48 hours in 5 µg/ml) facilitated use of this antibiotic, along with the cognate resistance gene (puromycin N-acetyl-transferase, PAC) for selection of Bge cells transformed with the PAC gene (*puroR*). Bge cells transfected with a plasmid encoding *puroR* were partially rescued when cultured in the presence of 5 µg/ml of puromycin. These findings pave the way for the development of functional genomic tools applied to the parasite-host interaction during schistosomiasis and neglected tropical trematodiases at large.

Keywords: *Biomphalaria glabrata***,** molluscan embryonic cell line (Bge), xCELLigence real time cellular analysis (RTCA), cytometrics, genetic transformation, antibiotic selection

1. Introduction

As the only known established molluscan cell line, the *Biomphalaria glabrata* embryonic cell line, Bge, represents a noteworthy resource for *in vitro* studies on the cellular and molecular basis of the relationship between the parasitic trematode, *Schistosoma mansoni*, etiological agent of the neglected tropical disease schistosomiasis, and its snail host (Knight et al., 2014). Recently there has been a rapid increase in "omics" based projects for both the parasite and snail that generated genomic and transcriptomic profiles (Raghavan and Knight, 2006; Berriman et al., 2009; Brindley et al., 2009; Chuan et al., 2010; Protasio et al., 2012; Zerlotini et al., 2013). In order to take advantage of the *in vitro* culture of the blood fluke intra-snail developmental stages, we considered that it was desirable to not only define cell characteristics of Bge cells, but also optimize functional genomic approaches to investigate the parasitism of the intermediate host by the parasite (Knight et al., 2014).

The snail cells or Bge conditioned culture medium have been employed to maintained *in vitro* intra-snail developmental stages of *Schistosoma mansoni* (Ivanchenko et al., 1999). Culturing miracidia in the presence of Bge cells facilitates the transformation, development and growth of sporocysts (Yoshino et al., 2013). Thus, transformed mother sporocysts, developing into daughter sporocysts *in vitro*, leading to the development of cercariae (the stage that is infective for the human host) has been accomplished (Coustau and Yoshino, 2000). The signaling networks operating between the snail and parasite interface can be more readily tracked by using either loss or gain of gene function tools applied to this *in vitro* co-culture system. Indeed, the genome sequence of *B. glabrata* currently being annotated will soon be released,

offering an opportunity to determine targets to silence and disrupt the *in vitro* development of the parasite. In order to perform cytometric and genetic transformation studies using the Bge cell line, we first established the growth behavior of the three isolates of Bge, known as SL, CB, and UK. The karyotypes of these isolates vary (Odoemelam et al., 2009); all exhibit aneuploidy, with modal metaphase chromosome complements of 63 and 67 for the SL and CB cell lines, respectively. These divergences confirm that these isolates have undergone substantial evolution in the laboratory since the Bge karyotype was first assessed and retained the expected 2n = 36 diploidly (Bayne et al., 1978).

Given the newly available draft genomes and the application of functional genomic tools, including RNAi and transgenesis technologies, and despite the difficulties on maintaining Bge cells, there is a pressing need to establish reproducible *in vitro* protocols to further develop these approaches for the molluscan cells (Yoshino et al., 2013). Earlier transfection studies using lipofection to introduce plasmids bearing either the *B. glabrata* or Heat Shock Protein (Hsp) 70 or cytomegalovirus (CMV) promotor into Bge cells, expressed the reporter gene luciferase (Lardans et al., 1996; Yoshino et al., 1998). Transgenesis in other mollusks, for example primary embryonic cells from the oyster *Crassostrea gigas* has also been accomplished by using a pseudotyped pantropic retrovirus expressing luciferase (Boulo et al., 2000). With the overarching goal of applying genetic manipulation tools to the Bge cell line, here we deployed, the xCELLigene Real Time Cellular Analysis (RTCA, Acea Biosciences) technology to examine cell growth in real time (Smout et al., 2010; Ke et al., 2011), and performed transfections of the cells with plasmids expressing fluorescent reporters and antibiotic resistant markers. These findings presented below pave the way for the development of functional genomic tools to

investigate parasite-host interactions during schistosomiasis and neglected tropical trematodiasis at large.

2. Materials and Methods

2.1. Bge cell line culture

Bge cell isolates SL (Sam Loker, University of New Mexico) and CB (Christopher Bayne, Oregon State University) have been described (Odoemelam et al., 2009). The cells maintained by one of us (JB) at Brunel University for 15 years were here termed 'UK isolate'. All cell isolates were cultured, maintained and passaged as described previously (Odoemelam et al., 2009). In brief, Bge cells were cultured at 25°C in air in filtered-sterile Bge medium (22% Schneider's *Drosophila* medium, 0.13% galactose, 0.45% lactalbumin hydrolysate, 14.1 µM phenol, gentamicin and 7% FBS), and passaged once a month using a cell scraper when confluence reached ~ 90% and re-seeded at 2 x 10^5cells/ml in plugged T25 flasks.

2.2. Puromycin dose response curve

The puromycin dose response curves were performed seeding 5x10^3 Bge cells per well into 6-well plates, and 2 days later puromycin dihydrochloride (Life Technology) was added to the culture medium to 0.05, 0.5 and 5 µg/ml medium. Given that puromycin is supplied at 10 mg/ml in 20 mM HEPES buffer (pH 7.2–7.5), cells cultured in10 µM HEPES (but without puromycin) served as controls. The cells were observed every day under bright light using a Zeiss Axio

Observer A.1 inverted microscope fitted with a digital camera (AxioCam ICc3, Zeiss). Manipulation of digital images limited to insertion of scale bars, adjustments of brightness and contrast, cropping and the like, was undertaken with the AxioVision release 4.6.3 software (Zeiss). The experiment was repeated three times.

2.3. Transfection of Bge cells

Bge cells cultured in 6-well plates were transfected as described (Knight et al., 2011) with either a GFP-encoding plasmid (pLenti-RNAi-GFP, Applied Biological Materials Inc) **(Figure S1A)** or a murine leukemia retrovirus plasmid encoding resistance to puromycin (pLNHX_puroR) derived from pLNHX_cHS4_650 (Suttiprapa et al., 2012) by replacing *neoR* with *puroR* **(Figure S1B)**. Briefly, using 4 μg of plasmid DNA and 3.12 μg of (N/P ratio=6) Poly Ethylene Imine (PEI *in vivo-plex* 'adherent'; AparnaBio), nanoparticles were formed in 200 μl of Bge medium incubated at 23 °C for 30 min (Knight et al., 2011; Liang et al., 2013). Following the addition of naked plasmid DNA/PEI nanoparticle complexes in 200 μl to the monolayer of Bge cells (80 to 90% confluent) in 1.8 ml Bge medium, plates (6-well plates) were maintained at 25 °C for 18 h in air before examining for fluorescence. To determine the efficiency and expression of GFP using PEI mediated transfection, we included analysis of similarly transfected HEK293T (human embryonic kidney) cells as a positive control. HEK293T after transfection were incubated at 37^0C in 5% CO_2. Both mammalian and Bge cells exposed to PEI only were included as controls.

2.4. Real time assessment by xCELLigence RTCA system of cell proliferation, antibiotic dose-

response curve and selection of transfected cells

Cellular proliferation of Bge cells, puromycin toxicity and selection of pLNHX_puroR-transformed Bge cells cultured in the presence of the antibiotic were assessed in real time using the xCELLigence DP system and E-plates (ACEA Biosciences, San Diego, CA), see http://www.aceabio.com/main.aspx (Ke et al., 2011). This real time cellular assay (RTCA) allows monitoring cellular events, e.g. cell proliferation and cell toxicity (cell death), by measuring the electrical impedance across interdigitated gold micro-electrodes integrated on the bottom of tissue culture E-plates. Bge cells were seeded in E-plates and assessment of proliferation and growth in real time assessed.

For the cellular proliferation assay of the Bge isolates termed SL, UK and CB, cells were collected using a cell scraper from the tissue cultured flask at 90% confluence and counted using Trypan Blue stain exclusion (0.4% Gibco Life Technologies) to determine cell viability. First, a background measurement was performed by establishing the Cell Index (CI) signal from the E-plate wells containing only 100 µl Bge medium at room temperature. Thereafter, 100 µl of cell suspension of 5,000, 10,000, 20,000 and 40,000 was inoculated per well (in duplicate) and the cells maintained at 23 °C for 30 minutes to allow them to settle at the bottom of the well. Cultures in E-plates were monitored in an xCELLigence DP platform for 160 hours with impedance measured at intervals of 15 minutes. Doubling times of the different Bge isolates were estimated in a time interval of 60 hours, i.e. from 20 to 80 hours after the addition of the cells, with the assistance of the RTCA Software 1.2 (ACEA Biosciences, San Diego, CA). For the puromycin dose-response curve analysis, 20,000 or 30,000 CB cells were seeded and

monitored as indicated above. When the average CI was ~ 0.7, the run was paused, 100 µl of cell medium was removed and replaced with 100 µl of medium containing 2X concentrated puromycin to reach the desired final concentrations, i.e. final concentrations in 200 µl per well in the E-plate, from 0.078 µg/ml to 5 µg/ml (2 fold-serial dilutions) or 10 µM HEPES buffer in Bge medium as vehicle control. The normalized CI obtained by dividing the CI value at each time point by the CI at the time of the puromycin addition, was calculated and employed to plot the dose response curve and to estimate the half maximal inhibitory concentration (IC50) of the antibiotic (RTCA Software 1.2, ACEA Biosciences, San Diego, CA) (Ke et al., 2011). To evaluate the selection of pLNHX_puroR-transfected Bge cells cultured in the presence of different concentrations of puromycin, 40,000 cells of the SL isolate were seeded per well and monitored as described above. When the CI reached a value between ~0.7 to ~1.0, the cells were transfected with (1) the plasmid pLNHX_puroR- PEI nanocomplex, or (2) PEI alone, following the protocol described above. A non-transfected, mock control was included. Forty- eight hours after transfection, 0, 2.5 or 5 µg/ml of puromycin was added to the culture medium and the cells were monitored for at least 84 hours after the addition of the antibiotic. The cellular growth was expressed as normalized CI (above) and as the percentage of the normalized CI (% normalized CI) of experimental groups in comparison to the mock control (% normalized CI = 100).

2.5 Statistical analyses

Levels of statistical significance among and between treatments were determined using Analysis of Variance (ANOVA) and Student's t-test. P-values of ≤ 0.05 were considered to be

significant. At least two biological replicates of each experiment were performed, each of which included two to four technical replicates, i.e. number of wells per group.

3. Results

3.1. Bge cells display a doubling time much longer than mammalian cells

In order to evaluate cell proliferation and quantify the doubling time of the isolates, real time cellular assay system (xCELLigence) was employed. Different numbers of cells of each Bge isolate ranging from 5,000 to 40,000 cells were seeded per well in the E-plates. The three isolates, SL, UK and CB proliferated in the E-plates displaying increasing CI values overtime (**Figure 1**). However, ostensible differences in the proliferative behavior, i.e. shape of the cell proliferation CI curve over time, among the isolates were evident. Interestingly, the increasing CI values were evident when more than 10,000 cells were seeded per well, and when 5,000 cells were seeded per well no incremental CI signals were detected in all the Bge isolates. In addition, remarkably differences in the estimated doubling times were detected among not only the three isolates, but also among the different numbers of seeded cells per well within each isolate. Bge isolates SL, UK and CB displayed doubling times longer than 40 hours ranging from either ~42 hours to ~157 hours or from ~ 41 hours to ~ 121 hours, when 40,000 or 20,000 cells were seeded per well, respectively. Despite these differences, the UK isolate exhibited the shortest doubling time ranging from 42 hours to 56 hours, when 40,000 to 10,000 cells were seeded per well (**Figure 2**). Concerning the cell size and morphology of the Bge isolates, the average diameter of these cells was ~18 µm, ranging from 14 to 24 µm and no significant differences in the morphology of the three strains were apparent (**Figures 1 and 3)**.

3.2. Bge cells are sensitive to the antibiotic aminonucleoside puromycin

The toxicity of the aminonucleoside puromycin (from *Streptomyces alboniger*), routinely employed for antibiotic selection of puromycin resistance marker-expressing mammalian cells (Ni et al., 2014), was investigated. Bge cells were sensitive to puromycin at from 50 ng/ml to 5 µg/ml for all three isolates, i.e. most of the cells (> ~90%) were dead between 2 and 5 days after the addition of the drug at 5 µg/ml and 50 ng/ml, respectively (**Figure 4**).

In order to quantify the cellular toxicity and the half maximal inhibitory concentration (IC50) of the antibiotic puromycin dose-response curves were performed using xCELLigence. Within less than five hours after the addition of 5 µg/ml of puromycin, the cell toxicity was evident and irreversible (**Figure 5A**). Additionally, at lower concentrations of the antibiotic, i.e. 0.2 and 1 µg/ml, the mortality based on the decreasing value of the normalized CI over time was evident. However, the Bge cells clearly recovered ~20 hours after the addition of low concentrations of puromycin (**Figure 5A**). The half maximal inhibitory concentration (IC50) of the antibiotic was calculated with the assistance of the xCELLigence RTCA Software 1.2. Dose-response curves were performed with concentrations of puromycin ranging from 0.078 to 5 µg/ml (2 fold-serial dilutions), and the normalization CI time point was chosen immediately before the addition of the antibiotic. **Figure 5B** shows the dose-response curves of Bge cells cultured in the presence of increasing concentrations of the antibiotic (i.e. normalized CI plotted against the log of puromycin concentrations) at different time points after the addition of puromycin. The IC50 of the antibiotic was $10^{-5.72}$ g/ml, i.e. ~1.91 µg/ml, for Bge cells under the conditions tested here.

The time dependent IC50 was stable from 24 hours after puromycin addition and remains almost constant for 3 more days **(Figure 5C)**.

3.3. A polyethylenimine (PEI)-based transfection protocol was effective to deliver reporter genes into BGE cells

Transfection protocols for Bge cells have been previously reported (Lardans et al., 1996; Yoshino et al., 1998). However, in the current study we employed for the first time, a recently described polyethylenimine (PEI)-based protocol for transfection of snails and *Schistosoma mansoni* schistosomules (Knight et al., 2011; Liang et al., 2013). Firstly, as proof-of-concept, mammalian cells were transfected following this protocol. The human kidney cell line HEK293T was transfected with the pLenti-RNAi-GFP plasmid encoding GFP driven by the CMV promoter **(Figure S1A)**, and 16 hours after transfection a strong GFP signal was evident in comparison to control cells **(Figure 6A-F)**. The GFP signal increased overtime and more cells displayed green fluorescence 48 hours after transfection (not shown). Second, we proceed to transfect Bge cells with the same plasmid encoding GFP and following the protocol successfully employed to transfect snails (Knight et al., 2011). GFP positive-Bge cells were evident five days after transfection in comparison to control cells exposed to PEI alone **(Figure 6G-L)**.

3.4. Bge cells transfected with a puromycin resistance marker partially rescued in the presence of antibiotic

Once the sensitivity profile of Bge cells to puromycin was established, along with an effective protocol to transfect the cells with plasmid DNA, we decided to investigate antibiotic selection and enrichment of Bge cells transfected with a puromycin resistant marker-encoding plasmid. Bge cells were seeded and grown in E-plates employing xCELLigence that allows the quantification of the effect (Rakers et al., 2014). The Bge cells were transfected with the plasmid pLNHX_puroR **(Figure S1B)** after they had reached CI values between ~0.7 and ~1.0. Two experimental groups were included; (1) cells transfected with the plasmid pLNHX_puroR- PEI nanocomplex, or (2) PEI alone, and a third non-transfected, mock control. Two days after the transfection puromycin was added to the culture medium at 0, 2.5 or 5 µg/ml and the cells were further monitored for at least 84 hours. The percentage of the normalized CI (% normalized CI) of experimental groups compared to the mock control (% normalized CI = 100) was calculated for the different puromycin concentrations, and plotted overtime **(Figure 7).** No significant differences among the three groups, i.e. the two experimental transfected groups and the control, were observed when the cells were cultured with 0 or 2.5 µg/ml of puromycin. However, a partial but significant rescue of ~40% was detected for cells transfected with both PEI and pLNHX_puroR and cultured for ~80 hours in the presence of 5 µg/ml of the antibiotic in comparison to control cells **(Figure 7A)**. The partial rescue of the transfected cells compared to control cells became apparent from ~2 days after the addition of 5 µg/ml of puromycin onwards **(Figure 7B).**

4. Discussion

From the time of its establishment in 1976 (Hansen, 1976) few laboratories have been able to

routinely maintain the Bge cell line for experimental use. Notwithstanding this complication, advances have been reported including the culture from miracidium to cercaria in the presence of Bge cells (Ivanchenko et al., 1999) and demonstration that Bge cells behave like hemocytes, the cellular mediators of the snail innate defense system (Humphries and Yoshino, 2006).

Three different isolates of Bge cells were analyzed in the current study; SL (Sam Loker, University of New Mexico) and CB (Christopher Bayne, Oregon State University) that were previously described (Odoemelam et al., 2009), and a third isolate maintained by us (JB and MK). Significant differences were not evident among the isolates in terms of morphology and size of the cells. However, marked variability in the doubling times among the isolates occurred, as quantified by the xCELLigence assay. The UK isolate displayed the shortest doubling time, ranging between ~40 hours to ~56 hours, and the most independent of the number of seeded cells. Remarkably, the doubling times of both SL and CB isolates were not only longer than the UK isolate, but also variable, depending on the number of cells seeded. CB displayed the longest doubling time (~157 hours), and inversely, SL displayed the shortest doubling time (~108 hours) when more cells were seeded. These differences are likely due to genetic changes to the three isolates that have independently evolved in culture. Indeed, karyotypes of CB and SL isolates had altered dramatically, revealing pronounced aneuploidy, from when this was first determined by Bayne et al. (1978). The karyotype remains to be determined for the UK cell isolate. However, based on these data showing that this isolate was the fastest to grow we could assume that its karyotype may also have changed in culture.

To date, considerable progress developing functional genomics tools for schistosomes has

been reported (Beckmann and Grevelding, 2012; Rinaldi et al., 2012; Mann et al., 2014). However, few if any reports have addressed functional genomics for *B. glabrata* or other intermediate host snails of schistosomes. Therefore, our results showing that Bge cells are sensitive to puromycin from 5 µg/ml to 200 ng/ml, for all three isolates, and quick kill time (2 days at 5 µg) is particularly encouraging. Also noteworthy is that this sensitivity enabled the moderate selection of cells harboring a plasmid expressing the puromycin *N*-acetyl-transferase, PAC gene, while cultured in the antibiotic. Transfection assisted with nanoparticles of PEI has been reported to deliver dsRNA and naked plasmid in snails and schistosomes (Knight et al., 2011; Liang et al., 2013), respectively, but is reported here for the first time for transient transfection of the snail cell line. PEI mediated transfection method was remarkably efficient and also non-toxic at the concentrations examined in both human and snail cell lines. Although a partial selection of the cells transfected with the plasmid bearing the PAC gene, driven by the 5'LTR of the MLV, was consistently observed under drug pressure, there was only a minor difference between survival of transfected cells and control non-transfected cells (rescue of ~40% was observed for cells transfected with both PEI and pLNHX_puroR in comparison to control cells). Reasons for this lower than expected selection could be that optimum function of the 5'LTR promoter occurs at 37^{o}C whereas Bge cells were maintained at 23^{o}C (room temperature). Furthermore, we used a heterologous virus promoter in these studies. To improve the selection, we are currently testing the use of a pantropic virus delivery system (Mann et al., 2014), and preliminary results are encouraging. To date, only one snail promoter, Heat shock protein (Hsp) 70 has been characterized and shown to drive the expression of the reporter luciferase gene (Yoshino et al., 1998). Following the availability of the genome sequence of this snail, which is pending (personal communication Coen Adema UNM, Pat Minx WashUGSC)

more snail promoters and cis-regulatory sequences likely will be characterized and optimized for genetic transformation studies.

With the findings presented here, characterizing the cytometrics, analyzing Bge cell sensitivity to puromycin, and subsequent use of the antibiotic to partially select the transfected cells, we have advanced prospects for facile efficient transfection of this cell line from the snail host of *S. mansoni*. We aim to transfer this technology to the intact snail, studying the sensitivity of this gastropod at large to antibiotics, including puromycin, to facilitate the eventual manipulation of the snail genome to dissect the molecular pathways that enable schistosomes (sporocyts) to develop *in vivo*. Although co-culturing of schistosome miracidia cultured with Bge cells can give rise to ceracariae (Ivanchenko et al., 1999), the method is inefficient. Primary cell lines derived from the gill tissue and hemocytes of the abalone mollusk, *Haliotis tuberculata* have been used to examine host pathogen interactions and also to test effect of toxins, respectively (Pichon et al., 2013; Minguez et al., 2014). Investigators in this field would benefit from access to techniques and protocols that optimize co-culture of schistosomes with Bge cells.

Mollusks and flatworms constitute the majority of lophotrocozoan taxa, yet only a single established cell line, the Bge cell line, exists from this super-phylum. This is in contrast to the wide application of insect cell lines, including from the lepidopteran *Spodoptera frugiperda*. The model Sf9 cell line from this moth, and baculovirus system, is used routinely for the production of functional recombinant proteins (Schneider and Seifert, 2010; Fogal et al., 2014). Cell lines from the fruit fly, *Drosophila melanogaster* are also widely employed for *in vitro* cellular and molecular studies, (reviewed by (Ceriani, 2007)), for example, mutagenesis of the S2 cell line

using the CRISPR/Cas9 gene editing approach has been recently accomplished (Bassett and Liu, 2014; Bassett et al., 2014). There is also a clear need for an established cell line in the schistosome-molecular tool-kit, and progress in this endeavor is being made, especially for *S. japonicum* (see review by (Ye et al., 2013)). Finally, the introduction here of the xCELLigence approach to analyze non-model, less-conventional cell lines, such as Bge of *B. glabrata*, is notable, and predicts follow-up studies to examine host/ parasite interaction in more detail such as in transwell/ co-culture experiments (Roshan Moniri et al., 2014). With the increasing availability of sequence information of the genome and transcriptome of both the snail host and the schistosome, it is opportune and timely to optimize Bge cells as companion 'tools' to provide more fundamental information on the schistosome/ snail host-parasite relationship, vector biology and indeed novel interventions. Since the snail is the natural obligate intermediate host for the schistosome parasite, discovery of mediators in the development of the in vitro co-culture of the parasite with the Bge cell line that we can interfere with can identify potential candidates for novel control strategies. All three Bge cell isolates utilized in this study are currently available, either as frozen stocks or in tissue culture from the NIH-NIAID SR3 repository at BEI (http://www.beiresources.org) in Manassas, Virginia, USA.

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Figure Legends

Figure 1. Cytometric analysis of the three Bge cell isolates. Cellular proliferation of Bge cell isolates SL (top left), UK (center left) and CB (bottom left) measured in real time by xCELLigence. The time interval employed to estimate the doubling times is indicated by the vertical bars (~20 hours and ~ 80 hours). Error bars: ±1SD of the mean. Representative images of Bge cell isolates SL (top right), UK (center right) and CB (bottom right). Magnification 20X, scale bars: 100 µm (top and center), 50 µm (bottom). The number of cells seeded per well is indicated in the top.

Figure 2. Doubling times estimated for the three Bge isolates. Doubling times of the Bge isolates were calculated in a time interval of 60 hours (from 20 to 80 hours after the addition of the cells, see Figure 1) for increasing numbers of cells seeded per well, with the assistance of the RTCA Software 1.2. Error bars: ±1SD of the mean. The maximum CI values reached by the three Bge isolates are indicated.

Figure 3. Bge cells in culture. Representative picture of Bge cells in culture. The diameter of the cells was estimated with the assistance of AxioVision release 4.6.3 software (Zeiss). Scale bar: 50µm

Figure 4. Bge cells are sensitive to the antibiotic puromycin. Representative images of Bge cells cultured in the presence of the indicated concentrations of puromycin, 2 days (top panel) and 5 days (bottom panel) after the addition of the antibiotic. Scale bars: 100 µm.

Figure 5. Dose-response of Bge cells to puromycin. Panel A: Cell proliferation measured by xCELLigence and shown as normalized CI over time of the Bge cells cultured in the presence of increasing concentrations of puromycin as indicated. Vertical bar represents the time point when the normalization was performed immediately before the addition of puromycin (arrow), i.e. ~79 hours after the cells were seeded, Panel B: Puromycin dose response curves generated at indicated time points after the addition of the antibiotic and employed to estimate the IC50 values with the assistance of RTCA Software 1.2. The mean IC50 is indicated, i.e. $10^{-5.72}$ g/ml = ~1.91 µg/ml, Panel C: Time dependent IC50 values from 24 to 72 hours after the addition of the antibiotic. The mean IC50 is indicated at ~1.91 µg/ml as the horizontal red dashed line.

Figure 6. GFP expression in cells transfected with the plasmid piLenti-RNAi-GFP and polyethylenimine (PEI). Panels A to F: Representative images of transfected human cells (HEK 293T) examined under bright field (A), fluorescent field (B) and merged (C). PEI only-transfected control human cells examined either under bright (D), or fluorescence field (E) and merged (F), Panels G to L: Representative images of transfected Bge cells examined under either bright (G), or fluorescence field (H) and merged (I). PEI only-transfected Bge cells examined under either bright (J), or fluorescence field (K) and merged (L). Scale bar: 100µm.

Figure 7. Partial rescue of Bge cells transfected with a puromycin resistant marker and cultured in the presence of the antibiotic. Cell growth is expressed as percentage of normalized CI (% Normalized Cell Index) of transfected cells compared to mock control cells (growth rate = 100%), and cultured in the presence of puromycin. Panel A: Cell growth in mock control, cells transfected only with PEI and cells transfected with both PEI and pLNHX_puroR, and cultured for 80 hours after the addition of 0, 2.5 or 5 µg/ml of puromycin as indicated. Panel B: Cell growth overtime after the addition of 5 µg/ml of puromycin in mock control, cells transfected only with PEI and cells transfected with both PEI and pLNHX_puroR. Error bars: ±1SD of the mean, * P-values ≤ 0.05.

Supplementary information

Figure S1. Constructs employed in the study. Panel A: Schematic of the plasmid pLenti-RNAi-GFP, Applied Biological Materials Inc, Panel B: Schematic of the murine leukemia virus plasmid pLNHXcHS4_650 encoding the neomycin resistant marker (left) (Suttiprapa et al., 2012) that was replaced with the puromycin resistant marker to engineer pLNHX_puroR (right). Maps are not as scale. MCS: Multiple Cloning Site

Figure 1

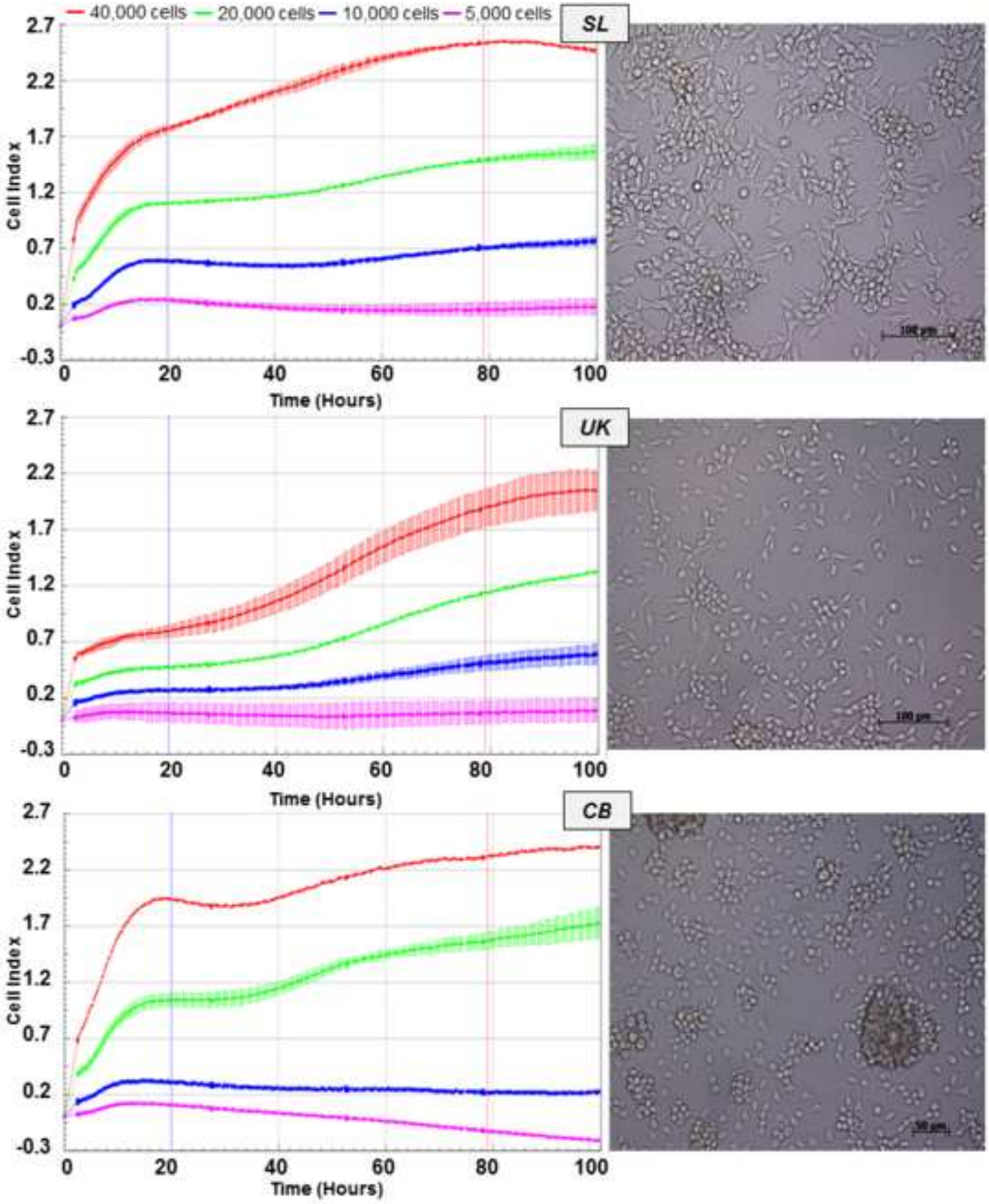

40,000 cells
20,000 cells
10,000 cells
5,000 cells
SL
UK
CB
Cell Index
Time (Hours)
2.7
2.2
1.7
1.2
0.7
0.2
-0.3
0
20
40
60
80
100

Figure 2

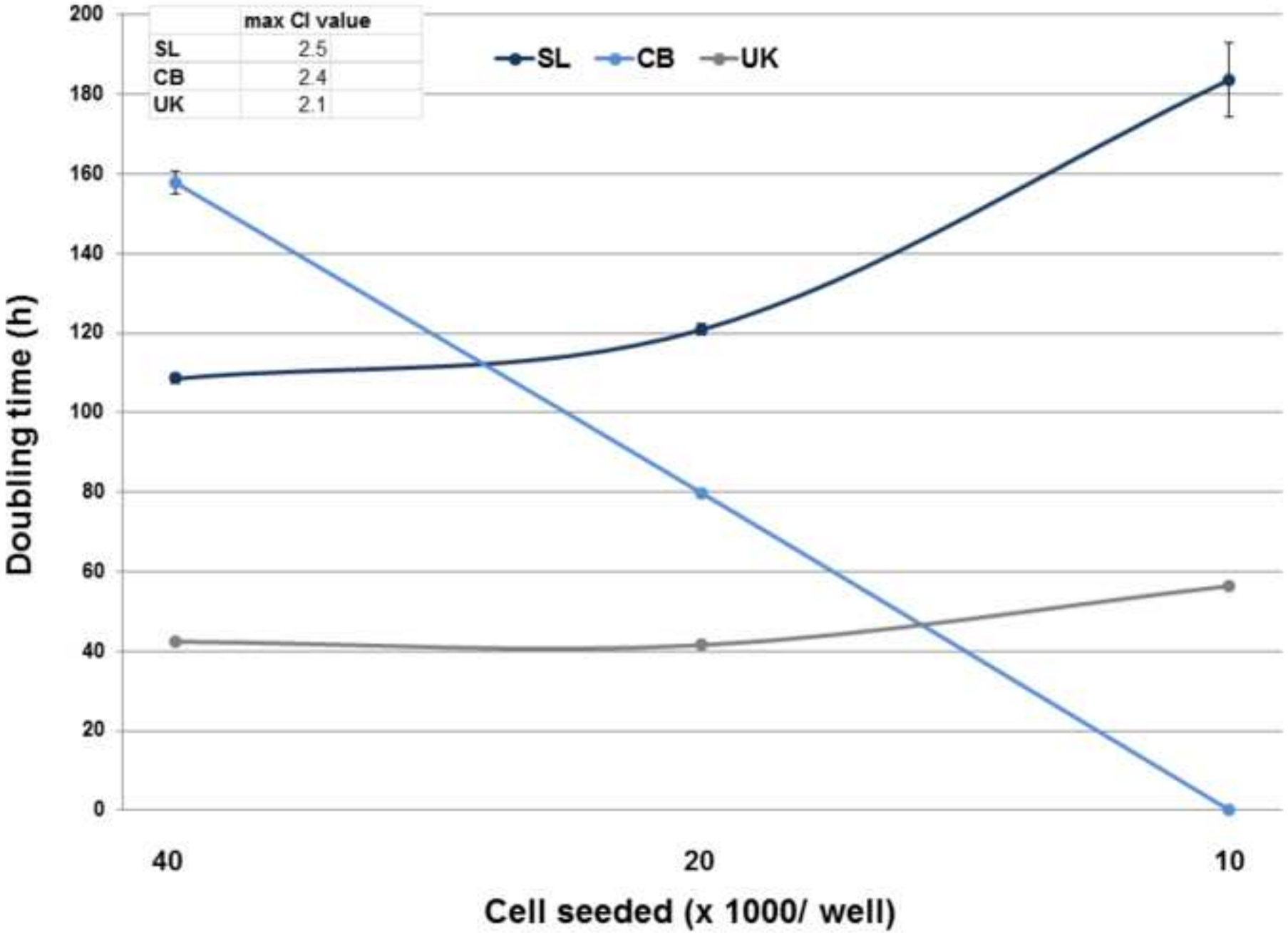

max CI value
SL 2.5
CB 2.4
UK 2.1
SL
CB
UK
200
180
160
140
120
100
80
60
40
20
0
Doubling time (h)
40
20
10
Cell seeded (x 1000/ well)

Figure 3

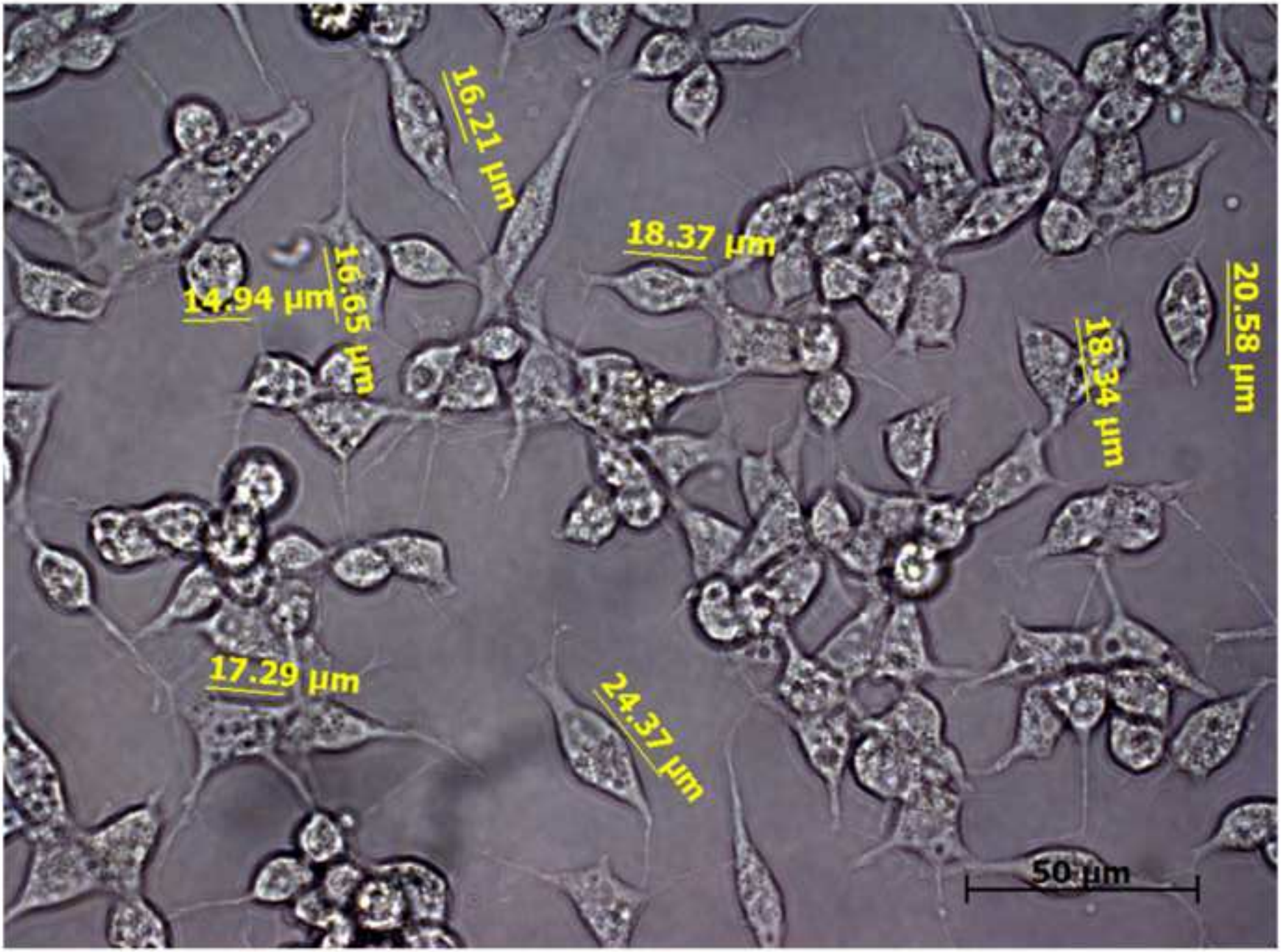

16.21 µm
18.37 µm
14.94 µm
16.65 µm
20.58 µm
18.34 µm
17.29 µm
24.37 µm
50 µm

Figure 4

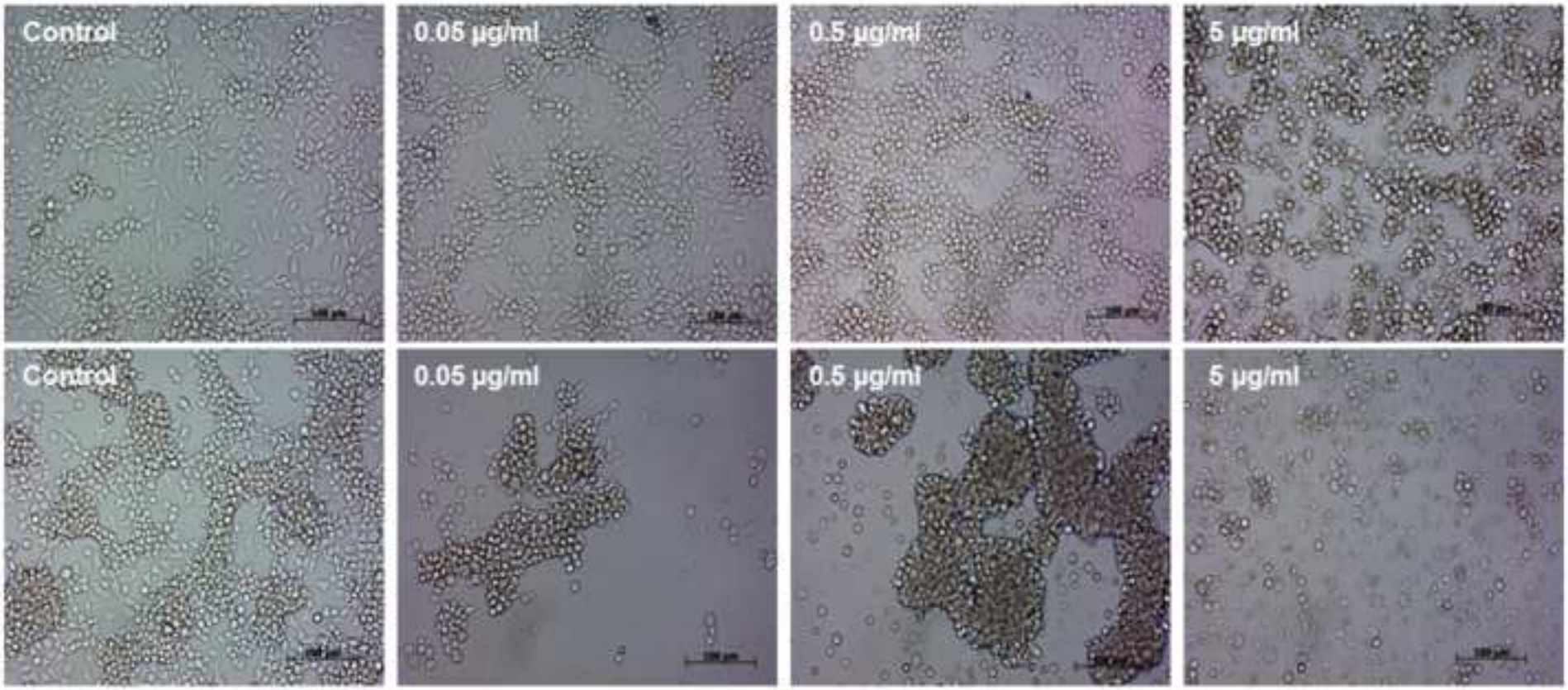

Control
0.05 µg/ml
0.5 µg/ml
5 µg/ml
Control
0.05 µg/ml
0.5 µg/ml
5 µg/ml

Figure 5

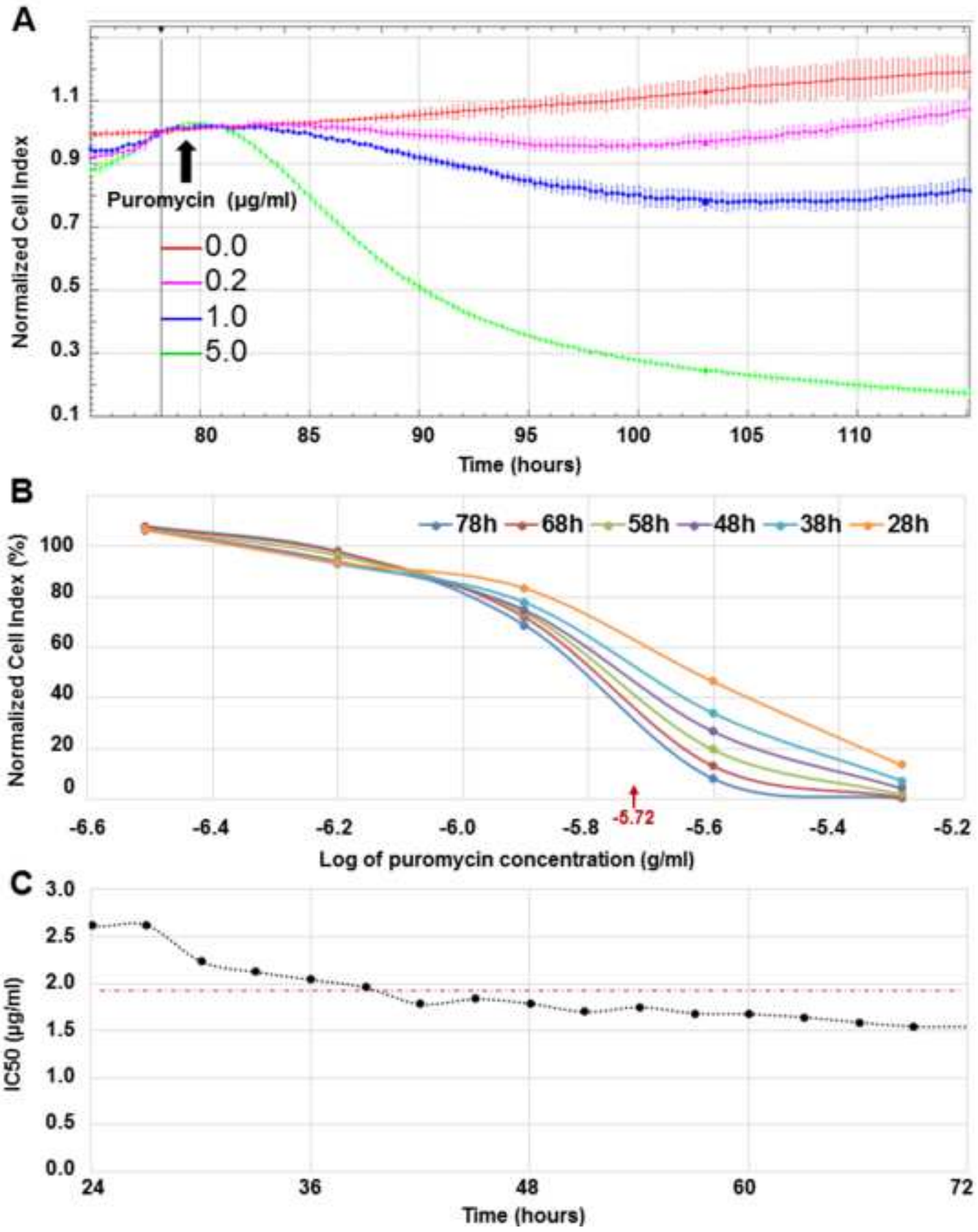

A
Normalized Cell Index
1.1
0.9
0.7
0.5
0.3
0.1
Puromycin (μg/ml)
0.0
0.2
1.0
5.0
80
85
90
95
100
105
110
Time (hours)
B
Normalized Cell Index (%)
100
80
60
40
20
0
78h
68h
58h
48h
38h
28h
-6.72
-6.6
-6.4
-6.2
-6.0
-5.8
-5.6
-5.4
-5.2
Log of puromycin concentration (g/ml)
C
IC50 (μg/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
24
36
48
60
72
Time (hours)

Figure 6

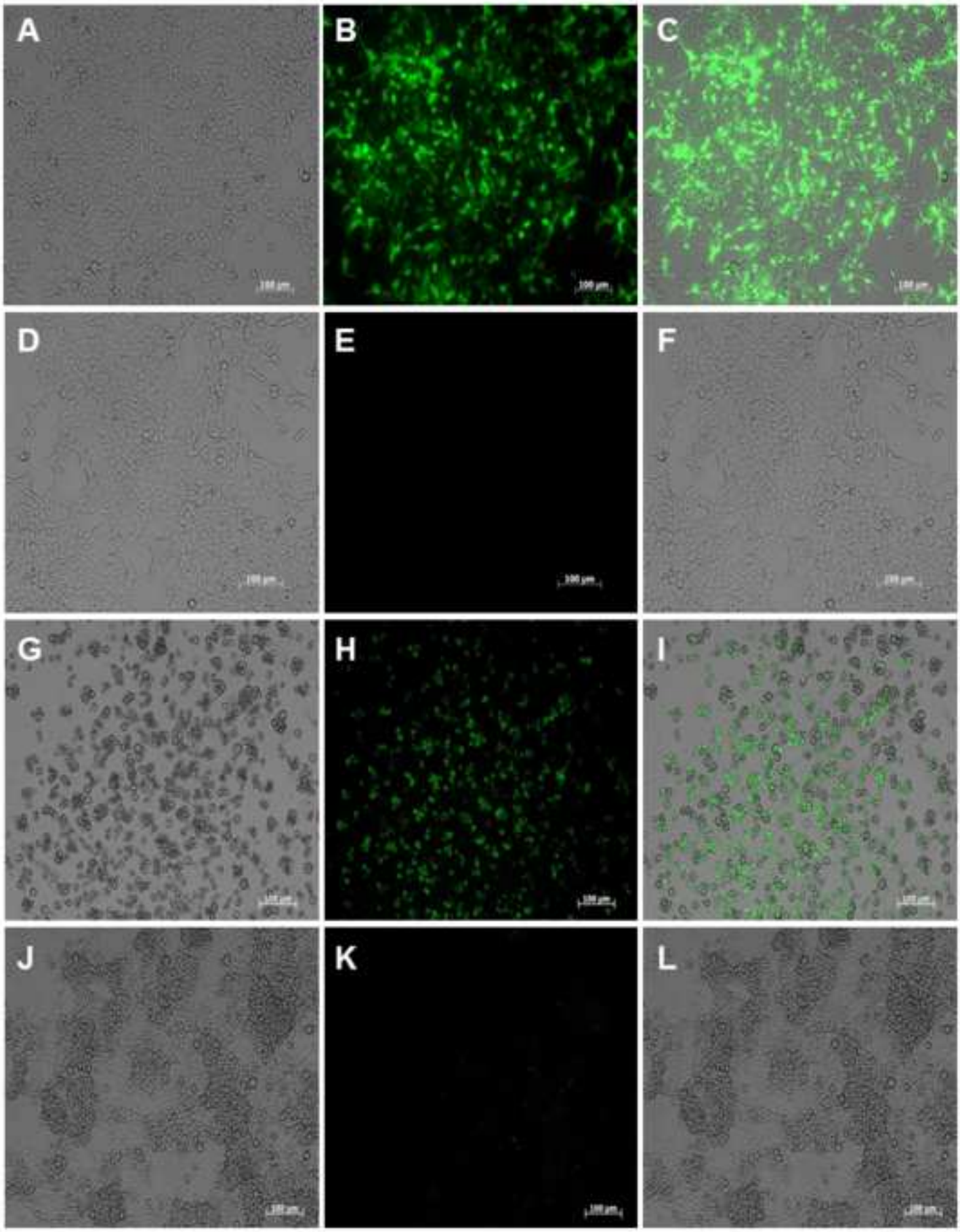
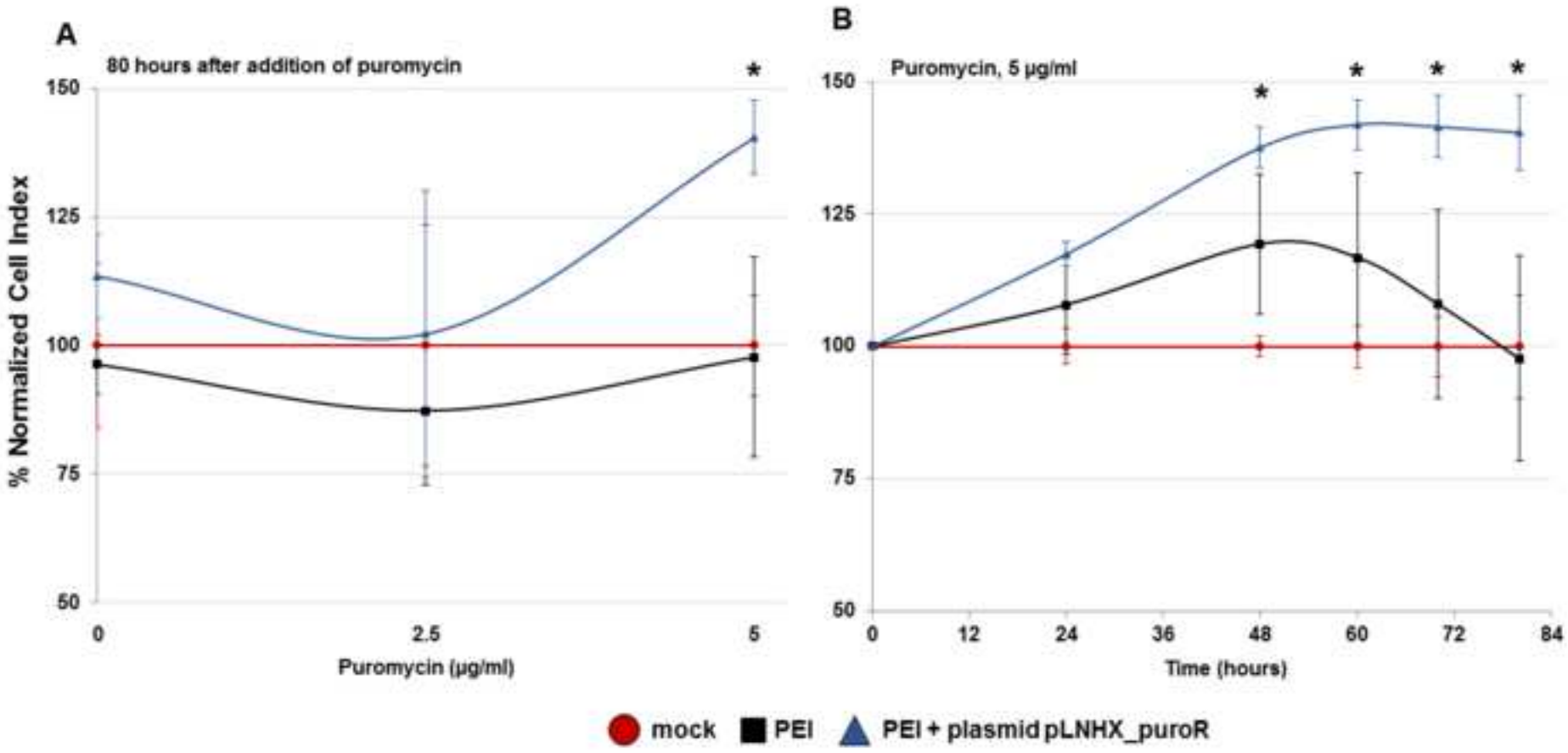


Figure 7


A
80 hours after addition of puromycin
% Normalized Cell Index
150
125
100
75
50
0
2.5
5
Puromycin (µg/ml)
*
B
Puromycin, 5 µg/ml
0
12
24
36
48
60
72
84
Time (hours)
mock
PEI
PEI + plasmid pLNHX_puroR

Supplementary Fig.1