

## CHAPTER III

### RESULTS

#### 3.1 Characterization of Cancer stem cell in Caco-2 cell line

The cluster of differentiation (often abbreviated as CD) is the molecules are markers on the cell surface, as recognize by specific sets of antibodies used to identify the cell type, stage of differentiation and activity of cells. Also in this study has been used CD 34 to verified the population of colon cancer stem cells that might be found in Caco-2 cell line. The Caco-2 cell line was purchased from ATCC and strictly maintained in the culture following the instruction manual provided by the agency. As can be seen in Figure 3.1 exhibit the histogram of Caco-2 cells CD34-FITC staining then counts by flow cytometry the red line represent auto-fluorescence of Caco-2 cells and the black line represent the Caco-2 cells were stained with antibody CD34-FITC. The result showed fluorescence intensity ship represent in the Caco-2 cell lines has small fraction of cancer stem cells. The cells about  $70\pm 7\%$  were positive staining with anti CD34-FITC.

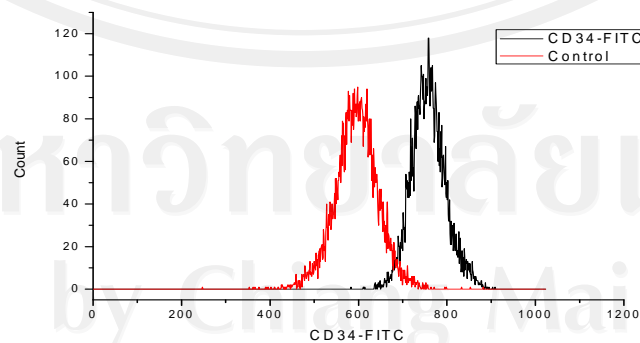


Figure 3.1 Histogram of Caco-2 cells staining with CD34-FITC then counts by flow cytometry the red line represent auto-fluorescence of Caco-2 cells and the black line represent the Caco-2 cells were stained with antibody CD34-FITC.

### 3.2 Real time monitors of Caco-2 cell growth behaviors in classical culture

#### **In the absence of doxorubicin:**

The Caco-2 cells were cultured under high resolution inverted light microscope combined with video imaging system. The video imaging of cells was recorded as function of time. As can be seen in Figure 3.2, in the absence of cytotoxic agent such as doxorubicin, immediately after addition cells into the system all cells were found in round shape suspended in the culture medium. After 8 hours of incubation, only small number of suspended cells was entering into the cellular kinetics since the division of cells was observed. At 24 hours later, majority of cells were adhered to the bottom of culture plate following differentiation to specific cell types. The real time imaging clearly demonstrated that the adhered cells presented very dynamic of plasma membrane and can be detached and changed to round shape following the division of cells before going to adhere at the bottom of culture plate. The real time video images also clearly demonstrated that these cells possessed pseudopodia-like or “sac” then the cytoplasmic liquid was transferred into the sac. Indeed the phenomenon facilitates the mobility of cell from one to other points. At 34 hours after culture, the cells clearly emitted white-blue light at the same time following a re-organizing the cell clusters (Figure 1d, e and f).



Figure 3.2 The Caco-2 cell behaviors in classical culture system in the absence of doxorubicin (a) the round shape of Caco-2 cells at immediately after cell were seeded. (b), (c), (d) cell was entering to cell cycle division. (e) The specific cells attached onto the bottom of culture plate. (f) Cell was detached from bottom become to round shape and then enter to division. (g) Cell was separated the nucleus and cytoplasm. (h) The completed cell division.

### 3.3 Kinetic monitor of drug response pattern in Caco-2 cell

#### In the presence of doxorubicin:

The pattern of Caco-2 cells responded to doxorubicin was clearly by using real time video imaging. As can be seen in Figure 3.3a, at 24 hrs after culture of 2D system, the Caco-2 cells were composed of (1) spindle-shaped cells attached to the bottom of the Petri dish and contacted to their neighbor cells formed monolayer of cells; (2) large irregular-shaped cells that attached both on the monolayer of cells and the bottom of Petri dish and (3) small round-shape cells that suspended over the monolayer of cells. Immediately, after addition 100  $\mu$ M doxorubicin into the system any morphological and behavioral changes of cells were observed. The changed in morphology and behavior was clearly seen at 1.30 hr after added the drug (Figure 3.3b). Most of cells became smaller in size but look healthy. Moreover, the culture surface where initially occupied by the monolayer of cells became loose and has free space. In fact, when the cells form monolayer they have cell-cell contact probably via the gap or tight-junction that linked the cells to each other. Even the cells have contraction induced by doxorubicin (chemotherapy drug) the membrane link of the cells still appeared (Figure 3.3c). After 5 hr of doxorubicin exposed, the cells were completely detached and suspended, the different of cellular morphology disappeared (Figure 3.3d). The cells became almost the same size and presented an actively movement of plasma membrane acting like cilia. It should be noted that the cells have differently chemosensitivity can be divided in three groups including the high, medium and low responsive group. The white light luminescence was clearly measured from the cilia. While the majority of cells were morphological and biochemical changed, a small numbers of cells did not.

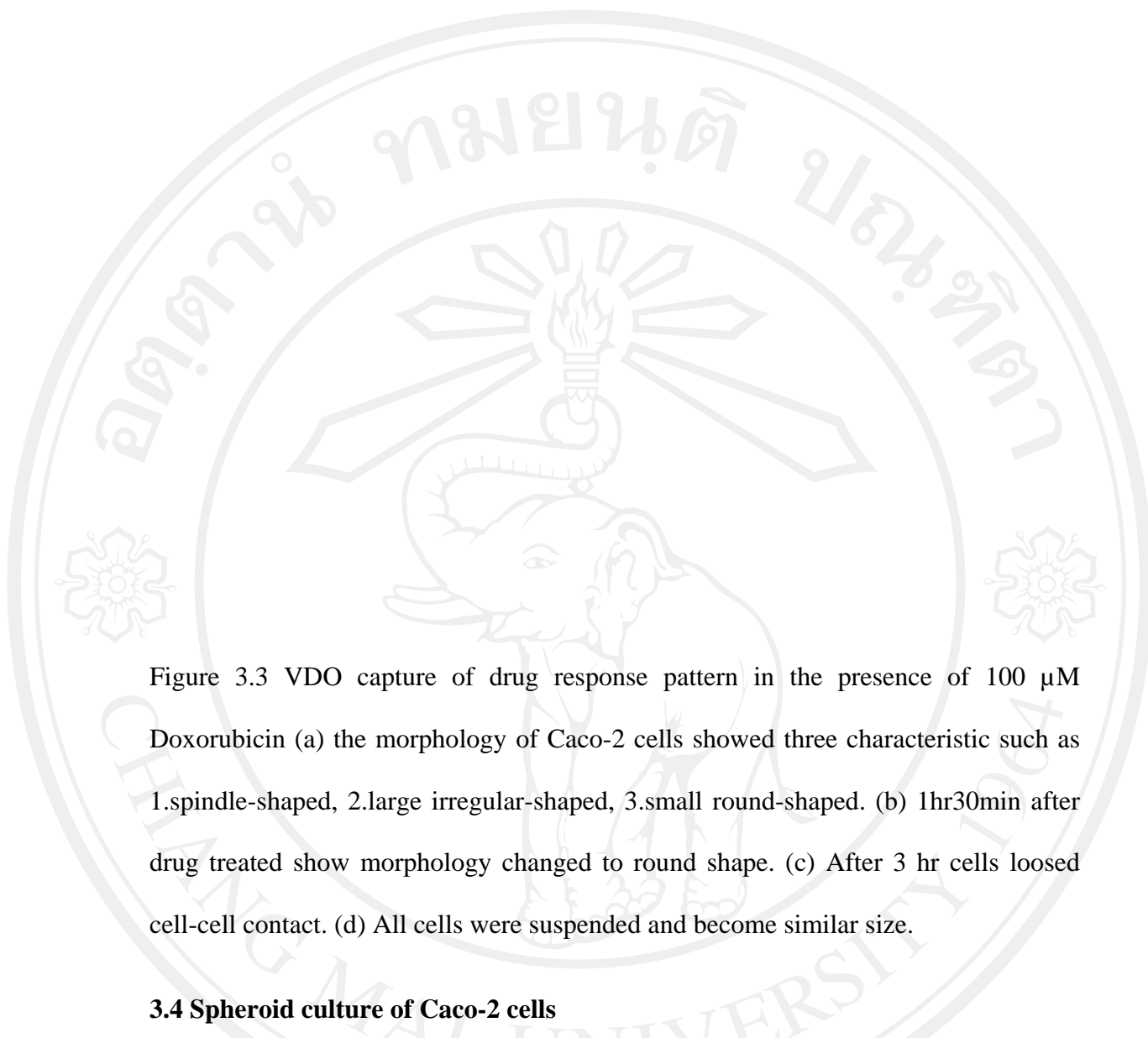


Figure 3.3 VDO capture of drug response pattern in the presence of 100  $\mu\text{M}$  Doxorubicin (a) the morphology of Caco-2 cells showed three characteristic such as 1.spindle-shaped, 2.large irregular-shaped, 3.small round-shaped. (b) 1hr30min after drug treated show morphology changed to round shape. (c) After 3 hr cells loosed cell-cell contact. (d) All cells were suspended and become similar size.

### 3.4 Spheroid culture of Caco-2 cells

Caco-2 cells were suspended in EMEM medium contained 7% acrylamide, gentle mixed and then ammonium persulfate and TEMED. Then the gel phase was form at room temperature where the cells were randomly embedded in side. The fresh EMEM medium was added in order to let the gel immersed into the medium. The culture systems were video imaged as a function of time. Caco2 cells can grow in the gel phase medium. As can be in Figure 3.4 a-d, at 20 days after culture, the spheroids were found throughout the culture system. The morphology of cells in the spheroid

can be visualized by staining the cells using a mixture of pirarubicin, acridine orange and rhodamine B (28). In fact, the color of light emission of acridine orange depends upon the microenvironments where the molecules were resided such as local pH and proteins. In an acidic compartments such as lysosomes, acridine orange give fluorescence in orange color, bright green in nucleolus and green color when interact with cytoskeleton protein. The viability of cells can be assessed by monitoring the intracellular organelle function including the mitochondrial energetic stage and lysosomal function by co-stained using rhodamine B, a mitochondrial probe (29) and acridine orange, a lysosomotropic (30,31)( Pourahmad et al., 2001). The nuclear compartments can be visualized by staining with pirarubicin. Figure 3.4c, d revealed more orange-red circles (lysosomes) and yellow circles (mitochondria) in cells. It was clearly demonstrated that the mitochondrial and lysosomal fluorescence was significantly difference from outer surface to the inside particularly at the mid core of spheroids these fluorescence dramatically declined signifying that the lowering cellular energetic stage. These suggested that the cells were probably undergoing death or in starvation stage.

Doxorubicin and pirarubicin are belonging to anthracycline antibiotic family and the kinetics of transport in cancer cells were well characterized (30, 32-35). Pirarubicin is a derivative of doxorubicin by which the hydroxyl group at carbon atom 4 of dosamine sugar was substituted by pyroran ring. It was reported that mean rate of passive diffusion of doxorubicin and pirarubicin into cells was  $0.01 \pm 0.003 \text{ pL}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$  and  $3.5 \pm 0.4 \text{ pL}\cdot\text{s}^{-1}\cdot\text{cell}^{-1}$ , respectively. The cellular concentration of doxorubicin was very low cannot be measured by using the methods used in this study. In the series of experiments, pirarubicin was used instead of doxorubicin. It

was reported that the uptake of pirarubicin by suspension cells was reached at steady state at 20 minutes. As can be seen in Figure 3.4, the cellular concentration of pirarubicin in spheroids, measured at 2 hrs after addition of the drug was very low compared with those in monolayer. Any changes in morphology and organelles function was not observed in spheroid conditions but clearly observed in monolayer.

Figure 3.4 Caco-2 cells were culture in spheroid condition and staining with molecular probe (10  $\mu$ M THP, 10  $\mu$ M Rho B, 5  $\mu$ M AO). (a) The green color of AO interacts with cytoskeleton protein. (b) The yellow color of Rho B represents the mitochondrial energetic stage. (c), (d) the orange-red circles represent lysosomes and yellow circles represent mitochondria



### 3.5 Tissue culture using 3D-PVDF nanofibrous scaffold system

Adding the egg-net design PVDF 3D-nanofibrous scaffold into the cell culture system dramatically changed the behaviors and facilitates the Caco-2 cells to form intestinal tissue. Figure 3.5 (a-d) indicated the typical results of the Caco-2 cells cultured in 3D-system, at 4 hrs after seeded, Caco-2 cells attached to the top surface of the scaffold without changing in morphology (figure 3.5a). The large-irregular shaped cells underwent differentiation and connected to another cells to form sheet of cells while the small round shaped cells attached on the sheet was clearly demonstrated at 72 h after culturing (Figure 3.5b). It should be noted that the sheet of Caco-2 became a three dimension of the tissue covered the scaffold (Figure 3.5c and d). The electron micrographs showed that Caco-2 cells can originate a tissue covered the top surface of the scaffold (Figure 3.6a and b) however, small-round shaped cells were found on the top surface of the tissue. The sheet of tissue presented micro-villi structures on the top surface (Figure 3.6c). The particular observation was that there were colonies of cells found on the tissue. The typical of electron micrograph of cross section of the scaffold was indicated in Figure 3.5d. The electron micrograph showed that tissue has thickness  $20 \pm 4 \mu\text{m}$  and some cells penetrated from the top side and migrated into the bottom side. Figure 3.6 (a-d) clearly showed that the ribbon and tubular structures were formed at the bottom side indicating that the cells can migrate from the top to the bottom side of the scaffold and then formed the basic structure of the tissue such vascular and nervous network.





Figure 3.5 the typical results of the Caco-2 cells cultured in 3D-system. (a) 4 hrs of Caco-2 spread on the top surface without changing in morphology. (b) 72 hrs cells were entering to differentiation resulting in irregular shape. (c), (d) the 3 dimensions of the tissue covered the scaffold.

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Figure 3.6 SEM micrograph of the intestinal mucosa tissue.