# CHAPTER IV

## DISCUSSION AND CONCLUSION

The Caco-2 cells were purchased from American type culture collection (ATCC) and were strictly maintained following the manual guideline of ATCC which is used in this study. In our experimental conditions, Caco2 cells were positively stained using anti CD34-FITC represent the fraction of cancer stem cells in the Caco-2 cells line. In previous studies is occur of side population cells in cell line (16, 25, 34). Study later exhibit the expression of putative stem cell markers on SP cells (5, 16, 35). We show the result of $CD34^{+}$ cells about 70.33 % in the Caco-2 cell line that obtained from ATCC together. That confirms the our hypothesis that in Caco-2 cell line have a small fraction of cancer stem cells reside in the cancer cell population. Later in the study of kinetic growth behavior in the classical culture show different pattern of two subpopulation of Caco-2 cells. The VDO imaging exhibit, group one is the large size and irregular shape cells after seeded on glass petri dish for 4 hour cells start adherent at the bottom. When culture later cell were expanded by extend the cytoplasm to contact another cells and form tight junction. Resulting to morphology changed some to be spindle and to be irregular shape. In contrast to the second group is the small size cells this group is very interesting because we observed cell division. At time 56 minutes cells start to enlargement follow separate nucleus, cytoplasm and completed division at 1 hour 24 minutes. These results suggested that Caco2 cell line comprise of cancer stem cells. This is consistency to those reported by Burkert et al. 2008 and Haraguchi et al 2005 (16, 24). The differential behavior of Caco-2 cells when cultured in conventional, spheroid and 3D-nanofibrous scaffold was rigorously investigated in

this study. For a series of experiments of the absence of cytotoxic drug: the results clearly showed that not all Caco2 cells entering to the cell cycle, only a fraction of cells did. It should be noted that in order to entering the cell cycle, the cells need to detach from their microenvironments to be suspension cells and became spherical shaped cells (figure 3.3 (a-h). The results also clearly demonstrated that the biomass of cells progressively increased to be twice of the beginning before undergoing both symmetric and asymmetric division. After the cellular division, the daughter cells attached onto the surface bottom of the Petri dish, formed a monolayer and expanded to free space nearby them. These cells were called as the cancer stem cells found in the Caco2 cell line. It was clearly found that when the density of cells was low, the membrane of Caco2 cells spontaneously and actively moved especially changed to be various forms. The cells were connected among them by the fibrous like structure (a part of plasma membrane) that should serve as intercellular bridges. The intercellular bridge length was varied from a few microns to at least 50-100 μm. The video images and electron micrographs clearly demonstrated that the cells spontaneously produced microvesicles that have 100 nm to 5 mm in diameter. These microvesicles were released and moved along the intercellular bridges from one cell to another cells. The cell-cell communication might be done via microvesicles and intercellular bridges by transferring of chemical signals between these cells. The real time video imaging also revealed that there was a synchronization of white, green or violet light luminescence among cells. We hypothesized that the phenomenon might indicate the network of cell communication since later the colonies following community of cells were formed. The similar results were reported for DU 145 human prostate cancer cells (36, 37) where the researchers suggested that in their conditions of experiments the

cells have deficient gap junction, the intercellular bridges may have a prominent role in transferring of chemical signals between these cells. By using time-lapse imagery, these authors reported that (1) filopodia rapidly move at a rate of microns per minute to contact neighboring cells and (2) intercellular bridges are conduits for transport of membrane vesicles (1-3 μm in diameter) between adjacent cells. Immunofluorescence detected alpha-tubulin in intercellular bridges and filopodia, indicative of microtubule bundles, greater than a micron in diameter (36, 37). For the experiments of the presence of cytotoxic drug, Doxorubicin consecutively induced the morphology changes. Drug response study was performed to confirm behavioral response of cancer stem cells to chemotherapy drug. The response pattern were clearly distinction the large cells (mature cancer cells) after treated with doxorubicin 100 μM cells was shrinkage to round shape (figure 3.4). After treatment and 5 h later the cells were detached and found as suspension cells (figure 3.4c). It is a mechanism of self-protection to avoid the cytotoxic agents. The sensitivity of cells to doxorubicin can be divided into 3 groups including high, medium and low sensitive group. It should be noted that the changes in morphology was observed only for the well differentiated while did not for the small round shaped cells or the cancer stem cells. The results revealed that cancer stem cells were high tolerance to doxorubicin compared with the well differentiated cancer cells. Small size cell population was still survived like cancer stem cells in the tumor. It becomes to quiescent state to protect them self and cause of cancer recurrent. Caco-2 behavior in 3D-gelECM it is very interesting we not observed the significance differentiation. Cells were poorly differentiated have round or spherical shape and aggregated like colonies of cells resided in the gel droplets (figure 3.6). Normally condition caco-2 cells adherent to bottom flask within 4 hour

and differentiate in 10 hour. It contrast to 3D gelECM culture we observe more than eleven hours but cells still have round shape. This behavior is likely to result from culture condition. In gelECM might be barrier of cells contact to medium. Resulting in cells starvation limited cell growth. This would demonstrate that used of gel as a ECM to culture would not be the suitable condition. We have new purpose of the 3D model constructed from nano-fibrous scaffold used in cell culture. The nano-fibrous scaffold is the flexible ECM it high porosity and elasticity. Thus the culture medium could be infiltrate to cells. Confirm by Caco-2 cell growth behavior on F6FU nano-fibrous scaffold. They can growth and differentiate to varies morphology. We observed fibrous-like structures that have microvessicles attached onto, wrapped around the nanofiber. These fibrous-like structures found to link the cells together and might be served as intercellular bridges when the cells were low density (figure 3.8d). The electron micrographs also indicated the ribbon-like and the tubular-like structures throughout the scaffold. It should be noted that these findings were found only when the cells were grown in the 3D culture system. This should be corresponded to the tubulogenesis the basically need of tissue development. Colas and Schoenwolf reported that the ribbon-like structures were the elongation of cells in the apicobasal dimension, with subsequent apical narrowing and basal expansion to create wedge shape cells that promote epithelial bending and wrapping (38). Wrapping occurs when an epithelial sheet curls until its edges meet and seal, forming a tubular structure. Typically, this involves only a portion of the epithelium, with the tube-forming cells first invaginating to form a crevice in the epithelium, then sealing off and separating from the rest of the epithelium, as during neural tube formation in many vertebrates. This generates a tube that runs parallel to the plane of the epithelium from which it

derives. Formation of tubes of the correct size and shape is essential for viability of most organisms, yet little is understood of the mechanisms controlling tube morphology. Many organs consist of epithelial or endothelial tubes that function to transport gases and nutrients to target tissues or to remove waste. While some organs are intricate networks of branched tubes, others are simple cylindrical tubes. Regardless of the final shape and size of a tubular organ, the cellular and molecular mechanisms by which a uniform sheet or cord of cells is transformed into a tube with polarized cells surrounding a central lumen are likely to be conserved among different organs of different species (39-41). Cancer stem cells in suitable condition they were able to display unique properties of cancer stem cells. The advantage of introducing 3D-nanofibrous scaffold in cell culture is to allow establishing the long term culture system until obtaining the complex intestinal tissue. This study is the first time reported that the Caco2 cells can originate the intestinal tissue which comprise the basic structure of the tissue such vascular and nervous network when cultured on PVDF scaffold. As the tissue has complexity mimic the mucosal intestinal tissue thus it should be suitable model for studying the interaction of anticancer drug in particular for colorectal adenocarcinoma.

**Conclusion**

The overal results of the study showed that the Caco2 cell line comprise of a fraction of cancer stem cells. These cancer stem cells preserved the characteristic of stem cells such as self renew and differentiation. It was possible to reconstitute the intestinal mucosa tissue by culturing the Caco2 cells in 3D culture system using egg-net design of PVDF 3D-nanofibrous scaffold. The advantage of the system are as follow: (1) the cell culture can be carried on for the desired time especially long

enough to have complet tissue and (2) the tissue samples can be easily handle and move to another systems for further studies. Importantly, these differences normally create a greater similarity between the cultured cells and the living organism (e.g. human being) the cells are meant to represent-leading to more useful data and more relevant research.