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參展科別　醫學與健康科學

作品名稱　葉酸缺乏及葉酸補充調控乳癌細胞轉化成癌幹細胞並促進體外腫瘤形成

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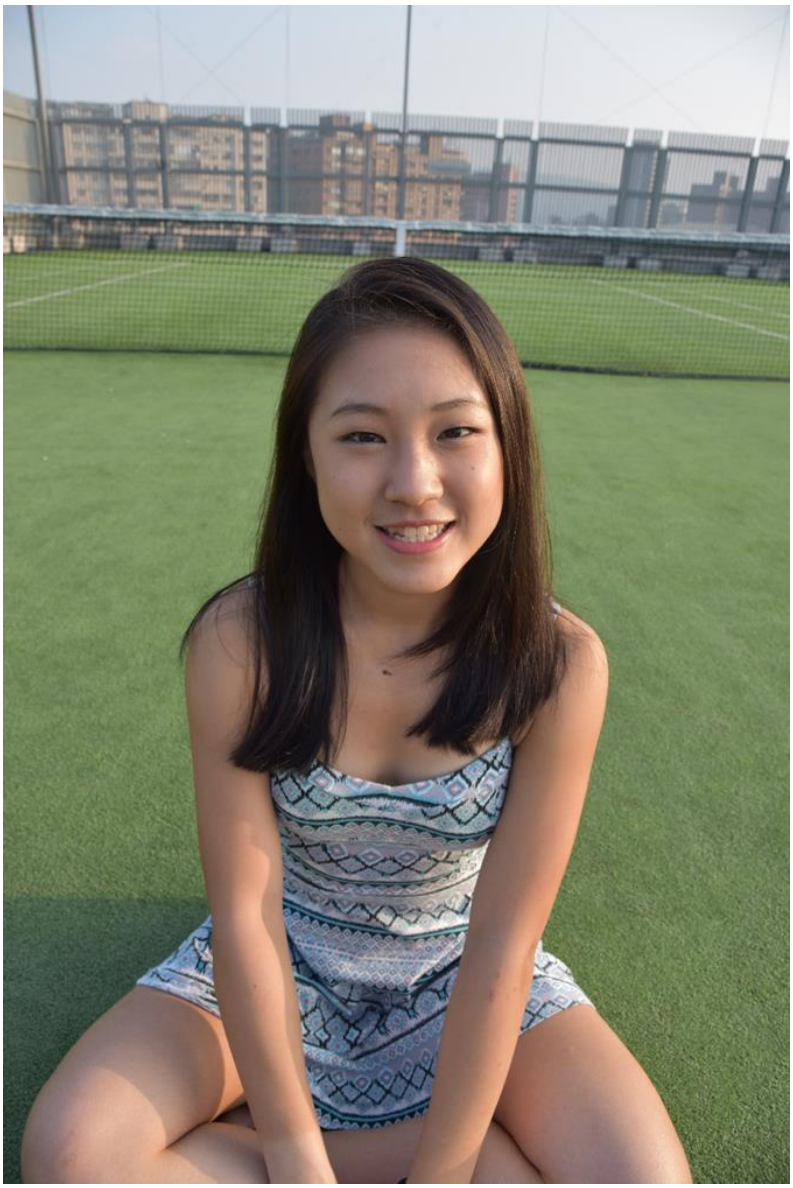
關鍵字　葉酸缺乏及補充、乳癌幹細胞、腫瘤球生長

作者簡介



我是黃得嘉 (Angel Huang) ，目前就讀台北美國學校高中部三年級。從小，我就喜歡自然科，尤其是生物和化學。上高中時，我發現實驗課程的樂趣，連續三年不間斷的實驗研究，全因為興趣以及好奇心。雖然經歷很多挫折，仍然持續勇往前進爭取更多學習及研究機會。非常感謝一直在我身邊支持陪伴我的家人與朋友及指導我的老師與教授們。

摘要

研究指出葉酸營養狀態與乳癌的風險有相互關聯且癌幹細胞是扮演癌症轉移的主要角色。葉酸缺乏和葉酸補充皆能促進癌細胞的轉移。本研究探討葉酸營養不良和葉酸補充是否會促進乳腺癌細胞轉變成癌幹細胞，將乳癌細胞株養於正常葉酸、低葉酸和高葉酸中，藉以模仿癌症病人的葉酸營養狀態。細胞在每個葉酸濃度中培養 2、 4 、 8 天後再培養於腫瘤球體培養液中並測量四種幹細胞標記-mTOR，SHH，Sox2 和 Nanog。結果顯示:低葉酸會導致癌細胞的生長停滯，藉由標記的表達增加顯示長期低葉酸會促進細胞轉變成癌幹細胞。於腫瘤球體培養液時，低葉酸組亦較對照組於體外產生較大及較多的腫瘤球。高葉酸組會加速癌細胞的增殖和向癌幹細胞的轉變且細胞增生較快並表達較多的標記，其腫瘤球體亦是最大和豐富的。總之，低葉酸和高葉酸都增強了乳腺癌細胞的腫瘤生成和轉變成癌幹細胞。

Abstract

Prescription of chemotherapeutic anti-folate drugs is the frontline treatment for breast cancer patients to induce folate-deficit cancer cytotoxicity. Alongside, folate supplements are used to reduce host toxicity. Few evidence has identified the exposure of cancer cells to low folate (LF) and high folate (HF) microenvironment can result in drug resistance and promote malignancy transformation, a cancer stem cell (CSC) phenotype. This study investigated whether imbalanced folate stress may promote CSC-mediated anchorage-independent tumorspheroid formation of breast cancer cells, the index of metastasis potential, and its underlying pathway. By western blot and flow cytometry analysis, cultivation of MCF-7 cells with LF and HF mediums promoted imbalanced folate metabolic stress, which coincided with increased expression of the pluripotent stemness markers (Sox2 and Nanog), stemness surface marker CD133, and epithelial to mesenchymal transition markers. Tumorspheroid assay revealed that both HF and LF exposure promoted self-renewal property of MCF-7 cells, evident by anchorage-indepdent tumorsphere formation. Quantitative proteomic analysis identified the changed complex signaling network signatures during transition of imbalanced folate cells to acquire CSC features in bioenergetics transformation, apoptotic and survival signaling, and upstream regulators of cellular migration and invasion. Our data for the first time demonstrated that imbalanced folate stress reprogrammed breast cancer cells into CSC to promote onco-spheroid formation with distinctive proteomic signatures of mediating their invasive and metastasis capability. The novel signatures could be used as therapeutic targets in the design of personalized nutrition-antifolate therapy for breast cancer.

壹、前言

一、研究動機

乳癌每年導致超過 50 萬人死亡;多於 90%的患者死於癌細胞轉移 (Jin and Mu, 2015)。轉移病灶損害生命器官，包含淋巴結，肺，肝，骨，腦，甲狀腺和心臟。乳癌轉移後，女性的中位生存率只有兩、三年 (Jin and Mu, 2015)。乳癌的異質性使得很難找到治癒和評估轉移的危險因素。 雖然已確定的乳癌的危險因素包括顯著的家族史，激素水平的改變，久坐的生活方式，高脂肪攝入的膳食和環境，乳癌轉移的分子因素仍不清楚 (Sharma *et al.*, 2010)。

二、研究背景及文獻回顧

(一) 癌幹細胞與乳癌惡性腫瘤進展之相關性

已有研究表明對化療有抗性的腫瘤細胞來自於與原始腫瘤的分子及表型不同的細胞亞群，這些細胞稱為腫瘤起始幹細胞或癌幹細胞 (cancer stem cells, CSCs)，而這些小部分的癌細胞能夠驅動腫瘤起始、增殖和擴散 (Liotta *et al.*,2001)。此理論是由 Lapidot 等人於 90 年代發表並解釋腫瘤的起源。根據 CSC 理論，CSCs 存在與其他幹細胞類似的方式生存: 在具有低氧氣濃度的微環境中以聚合狀態來適合生存，並且於細胞週期 G0 保留更常時間因以延長它們的壽命 (Lapidot *et al.*,1994)。這使 CSC 更容易突變或基因/表觀基因地改變。這細胞送發指示使癌幹細胞群進行自我更新以維持細胞群並分化產生腫瘤 (Cukierman and Bassi, 2012)。

Maenhaut 等人指出 CSCs 的三個主要特徵: 自我更新的能力，重建原腫瘤的完整癌細胞的能力，以及不同的表面生物標誌的表現 (Curtin and Lorenzi, 2010)。幹細胞具有自我更新的能力是非常罕見的，因為其最基本的條件為一個聚集 (niche) 同時具有支持(上皮或基質）細胞，細胞外基質，血管和神經纖維，故導致大多數幹細胞維持休眠狀態雖有增殖的潛力 (Maenhaut *et al.*,2010)。不需依附 (anchorage-independent) 的腫瘤球體 (tumorsphere) 的形成是細胞自我更新能力的特徵 (Cao *et al.*,2011)。Velasco-Velazquez 等人發現，當乳腺癌細胞

轉化為 CSCs 時，它們具有不對稱分裂的能力，並產生能夠自我更新的幹細胞以及能夠產生癌細胞的異源譜系細胞 (Velasco-Velázquez *et al.*, 2011)。

轉分化(transdifferentiation)是癌幹細胞的另一個指標 (Tang, 2012)。癌幹細胞會先由上皮細胞型態轉變成具移動性的間葉細胞的狀態; 此過程稱為表皮-間葉轉換 (Epithelial-mesenchymal transition, EMT) (Shen, Burke, and Tosh, 2004)。

EMT 透過 EMT 誘導轉錄因子 (EMT-inducing transcription factors, EMT-TFs) 控制細胞與細胞接觸、細胞極性、細胞骨架及細胞外基質降解的蛋白質表現。在此過程中，上皮細胞基因被下調，由間充質 (mesenchymal) 的蛋白質取代。主要上皮生物標誌物包含 *E-cadherin*、*cyctokeratin*、*occluding* 及 *ZO-I;* 主要間充質標記包含 *matrix metalloproteinases (MMPs)*、*N-cadherin*、*fibronectin* 和 *vimentin*。不論直接或間接因素導致 *E-cadherin* 表達的減少是 EMT 中最關鍵的一個步驟。(Puisieux, Barbletz, and Caramel, 2014)

音猬因子 (*sonic hedgehog, SHH*) 的信號調節細胞增殖和分化時 EMT 的互動 (Strickland, Krupenko, and Krupenko, 2013)。EMT-TF 可減輕細胞對腫瘤的抑制機制，導致細胞轉為惡性及發展成腫瘤 (Mukherjee *et al.*,2006)。來自上皮組織的 CSC 顯示出像胚胎幹細胞之生物標誌物，包含癌基因 *c-Myc* 和多能性因子如 *Sox-2* 和 *Nanog* (Hadjimichael *et al.*,2015)。*mTOR* 途徑在許多細胞過程會被激化，尤其是腫瘤的形成，並被認為癌症的主調節物 (Xia and Xu, 2015)。*mTOR* 信號維持 CSCs 的自我更新以及癌變的功能 (Xia and Xu, 2015)。因此，CSCs 的增加會誘導 *SHH* 和 *mTOR* 途徑以及 *Sox-2* 和 *Nanog* 的蛋白表現。此推論表明腫瘤球的形成可以表達 CSC 的表行(phenotype) 並引起乳癌的轉移 (Xia and Xu, 2015)。

(二) 乳癌治療之化學治療(化療)

長期以來，化療已被公認為乳癌的前線治療策略。但是，癌細胞的多藥耐藥性導致化療無法保證成功 (Chiarugi *et al.*,2012)。

癌細胞的耐藥能力來自於惡性腫瘤中的 CSCs。最常見的化療和放射線治療方法，減少了腫瘤塊中快速生長的癌細胞;但它不會防止復發，表示治療無法移除 CSC。相反的，研究已指出這些療法其實對 CSC 的生長有幫助。很多時候，晚期癌症雖然對最初的治療有反應，之後會復發。(Sun *et al.*,2015)

對於乳癌,患者通常接受化療的抗葉酸藥物 (Eyler and Rich, 2008)，這些藥物限制葉酸的產生，然而，葉酸對一些依賴葉酸的酶及細胞生長過程,尤其是單碳代謝是特別重要。

單碳代謝介導體內 DNA 的合成因為它產生嘌呤和胸苷酸並將同型半胱氨酸再甲基化形成甲硫氨酸 (蛋白質合成的必需氨基酸)。葉酸在單碳代謝中扮演輔酶的角色；然而，因為葉酸依賴性酶和葉酸結合蛋白的濃度遠遠超過葉酸輔因子的量，葉酸輔因子是代謝週期中的限制因素。(Stover, 2009)

化療引起葉酸缺乏（folate deficiency, FD），產生低葉酸代謝壓力（low folate metabolic stress, LFMS）(Zhao, Matherly, and Goldman, 2009)。 LFMS 被認為是促進癌變作用的表觀遺傳修飾因子和致突變因子 (Visentin, Zhao, and Goldman, 2013) 。為了對抗葉酸缺乏，葉酸補充劑常被用於平衡葉酸狀態並防止與抗葉酸藥物相關的症狀 (Stockstad, 1990)。最近，少數人指出葉酸補充劑實際上增加癌症的風險，因為它們促進腫瘤前期和未診斷腫瘤病灶的發展 (Wien *et al.*,2012)。先由 Sidney Farber 在 1940 年代觀察到，葉酸補充也可以促進癌細胞的葉酸代謝和促進增殖 (Yi, 2007)。然而，葉酸補充和癌症的爭議仍未被澄清。因此，確定葉酸缺乏及補充是如何影響癌細胞增殖和腫瘤的生長機制是至關重要的。

(三) 研究目的及研究的問題

本研究假說為葉酸營養不良及補充調節乳癌細胞轉化為癌幹細胞及促進體外腫瘤形成。其研究目標探討如下:

1、葉酸缺乏及補充是否影響乳癌細胞的生長。

2、葉酸缺乏及補充是否促進乳癌細胞轉化成癌幹細胞及體外腫瘤形成。

3、葉酸缺乏及補充是否調控乳癌細胞生物標記的表現。

貳、實驗材料與方法

一、實驗材料

(一) 細胞株

由 American Type Culture Collection (ATCC)購得乳癌細胞 MCF-7 (Michigan Cancer Foundation-7, ATCC HTB-22, Manassas, VA, USA) 。

(二) RPMI 1640 培養液

完整配方 Roswell Park Memorial Institute (RPMI)1640 (Gibco Lab, Grand Island, NY, USA) 添加 10% 胎牛血清(FBS, Gibco Lab)、0.2% 碳酸氫鈉 (NaHCO3, Sigma, St. Louis MO)、 100 units/ml 青黴素(penicillin, Sigma, St. Louis, MO, USA) 、100 units/ml 鏈黴素(streptomycin sulphate, Sigma, St. Louis, MO, USA) ，調整 pH 值至 7.4，過濾後存放於 4℃。此培養液的葉酸濃度為 2.2μM。

(三) 低葉酸 (Low folate, LF) 培養液

RPMI1640 (without folic acid, 0.2%$NaHCO_3$, Gibco Lab) 添加 10% FBS、100 units/ml 青黴素、100 units/ml 鏈黴素、以及 10nM 葉酸，調整 pH 值至 7.4，過濾後存放於 4℃。此培養液的葉酸濃度為 2.2 μM。

(四) 高葉酸 (High folate, HF) 培養液

RPMI1640 (without folic acid, 0.2%$NaHCO_3$, Gibco Lab) 添加 10% FBS、100 units/ml 青黴素、100 units/ml 鏈黴素、以及 50μM 葉酸，調整 pH 值至 7.4，過濾後存放於 4℃。

(五) 腫瘤球體培養液 (oncospheroid medium)

Dulbecco's Modified Eagle Medium-F12 (DMEM-F12, Gibco Lab) 培養液添加 100 units/ml 青黴素、100 units/ml 鏈黴素、2% B27 (Thermo, Rockford, IL, USA) 、10 ng/ml Recombinant Human Fibroblast Growth Factor (rhEGF, Sigma) 、20 ng/ml Recombinant Human Epidermal Growth Factor (rhEGF, Sigma) ，過濾後存放於 4℃。

二、儀器設備

CO_2 培養箱、倒立式顯微鏡、西方墨點法電泳及轉漬設備、酸鹼測定儀、恆溫水浴槽、微量離心機、照相膠體系統、加熱板、BioSpectrum Imaging System 照相系統 (UVP, Upland, CA, USA)，Celígo (Nexcelom, Lawrence, MA, USA) 。

三、實驗方法

(一) 細胞培養

乳癌細胞 MCF-7 培養於 RPMI 1640 培養液，置於含 5%CO_2 及飽和水蒸氣之 37℃恆溫培養箱，每隔 3 天進行換取培養液或繼代培養。

1、對照組

將 MCF-7 細胞培養於 RPMI 1640 培養液置於含 5%CO_2 及飽和水蒸氣之 37℃恆溫培養箱，依照實驗不同時間點收取細胞進行分析。

2、低葉酸

將 MCF-7 細胞培養於低葉酸培養液置於含 5%CO_2 及飽和水蒸氣之 37℃恆溫培養箱，依照實驗不同時間點收取細胞進行分析。

3、高葉酸

將 MCF-7 細胞培養於高葉酸培養液置於含 5%CO_2 及飽和水蒸氣之 37℃恆溫培養箱，依照實驗不同時間點收取細胞進行分析。

(二) 細胞融合測定 (Confluency assay)

將培養於正常葉酸、低葉酸及高葉酸培養液 2 天、4 天及 8 天的 MCF-7 細胞，以胰蛋白酶 (Trypsin, Sigma) 收取細胞，使用血細胞計數板及台盼藍 (Trypan Blue, Sigma) 計數2×10^5顆細胞後種植於 96 孔 (96-well) 盤 (Corning, Corning, NY, USA) 以細胞各自的培養液培養 4 天。每一天同一時間使用 Celígo (Nexcelom, Lawrence, MA, USA) 偵測細胞融合指數。

(三) 腫瘤球培養

將培養於正常葉酸、低葉酸及高葉酸培養液 2 天、4 天及 8 天的 MCF-7 細胞，以胰蛋白酶 (Trypsin, Sigma) 收取細胞，使用血細胞計數板及台盼藍 (Trypan Blue, Sigma) 計數 5000 顆細胞後種植於極低貼附 6 孔盤 (Corning, Corning, NY, USA)中，以腫瘤球體培養液培養 3 天、6 天及 9 天後，觀察腫瘤球數量及型態。

其中，培養於正常葉酸、低葉酸及高葉酸培養液 2 天的細胞腫瘤球使用 Celígo 的腫瘤球體測定，其提供腫瘤球數目、平均體積、平均面積及平均周長。

(四) 西方點墨法

1、全蛋白萃取

將細胞培養液移除，以磷酸塩缓冲生理鹽水(phosphate buffered saline, PBS, Sigma)沖洗 2 次，加入 1 倍細胞裂解缓冲液(lysis buffer)。此配方為 10% 10 倍 lysis buffer (Cell Signaling Technology, Boston, MA, USA), 10% 100 倍蛋白酶抑制劑(Protease inhibitor, Thermo)及 1%苯甲基磺酰氟(phenylmethylsulfonyl fluoride, Thermo) 。利用細胞刮杓刮下細胞收取至 eppendorf 管，置於冰上作用 15 分鐘，以 13200 rpm，4℃ 離心 30 分鐘，取上清液即為全蛋白質萃取液，保存於-20℃。

2、蛋白質定量

使用 BSA protein assay kit (Bio-Rad, Hercules, CA, USA) 偵測蛋白質濃度。先將 5x Bio Red protein assay 試劑以 1:4 與去離子水稀釋。在 96 孔盤內，每孔加入 200μl 的稀釋後試劑分別與不同濃度 之 Bovind serum albumin standard (BSA) 標準品 (12.5, 62.5, 125, 250, 375, 500 μg/ml) 及稀釋過蛋白質萃取樣品混勻反應 5 分鐘，以 Elisa Reader (Dynatech Laboratories, West Sussex, UK)，波長 595nm 測定其吸光值。將各濃度標準品數值作為一條標準迴歸曲線，將蛋白質萃取樣品之吸光值代入標準曲線計算樣品蛋白 質濃度以及定量 40 μg/μl 的蛋白量。

3、蛋白質電泳

定量之蛋白質萃取液與 6X dye (Thermo)混和，100 ℃乾浴加熱 10 分鐘後置冰上冷卻 10 分鐘，加入 SDS-polyacrylamide page，以上膠 80V 30 分鐘、下膠 100V 75 分鐘進行電泳。

4、轉漬

取下電泳完成之膠片，於轉漬卡匣依序疊放沾濕海綿、濾紙、膠片、 PVDF 膜 (Thermo) ，需先浸泡甲醇活化，濾紙、海綿，夾緊並放入電泳轉漬槽，以 100V 轉漬 45 分鐘，使膠體的蛋白質轉印到 PVDF 膜。

5、免疫反應與呈色

將轉漬完成的 PVDF 膜已 5% 脫脂牛奶 (Anchor, Taipei, Taiwan)進行阻攔，反應時間為 30 分鐘室溫 2 次於 75 rpm 的振動器，再以 TBST (Sigma) 75 rpm 室溫清洗 5 分鐘共 3 次。清洗後，加 入一級抗體 Actin (Santa Cruz Biotechnology, Santa Cruz, CA)、 SHH (Santa Cruz Biotechnolgoy)、Sox-2 (Genetex, Irvine, CA)、Nanog (Genetex)、E-cad (BD Biosciences)、Vimentin (Cell Signaling Technology)及 mTOR (Genetex)，以 1:500~1:2000 稀釋於 TBST，4℃反應 18 小時。移除一級抗體，使用利用 TBST buffer，55 rpm 室溫清洗共 3 次，加入二級抗體 anti-rabbit IgG antibody (Genetex) 1:2000 及 anti-mouse antibody (Genetex)

1:2000-1:8000 稀釋於 5% blocking buffer， 室溫反應 1 小時。最後 PVDF 膜利用 TBST buffer，55 rpm 室溫清洗共 3 次，PVDF 膜與 SuperSignal West Femto Chemiluminescent Substrate (Thermo) 顯影劑反應，使用 UVP BioSpectrum Imaging System (UVP, Upland, CA, USA).

四、統計分析

實驗數據採用 SAS 9.4 套裝軟體 (SAS Institute, Inc., Cary, NC, USA) 進行統計分析。使用 One-way ANOVA 分析各組間差異以及 Duncan's Multiple Range Test，以 $P < 0.05$ 達統計上顯著差異。

參、研究結果

一、低葉酸和高葉酸的培養調控 MCF-7 細胞的生長

在對照培養基中培養 MCF-7 細胞在 72 小時之前達到 80%融合（圖 1），從 0 小時穩定增加。72 小時後，融合水平開始下降（圖 1），這表示細胞已從細胞培養板分離並死亡。對照培養基中的 MCF-7 細胞具有約 35.49 小時的平均倍增時間。與對照相比，在低葉酸培養基中生長的細胞甚至在 96 小時後也沒有達到 80%融合（圖 1）；並且在 48 小時後生長速度開始穩定。平均來說，低葉酸培養基中的細胞其倍增時間約為 49.20 小時。相反，用高葉酸培養基處理的 MCF-7 細胞在 48 小時內達到 80%融合，然後穩定地由緩慢至無生長直至 96 小時（圖 1）。這些細胞的平均倍增時間為 19.04 小時。

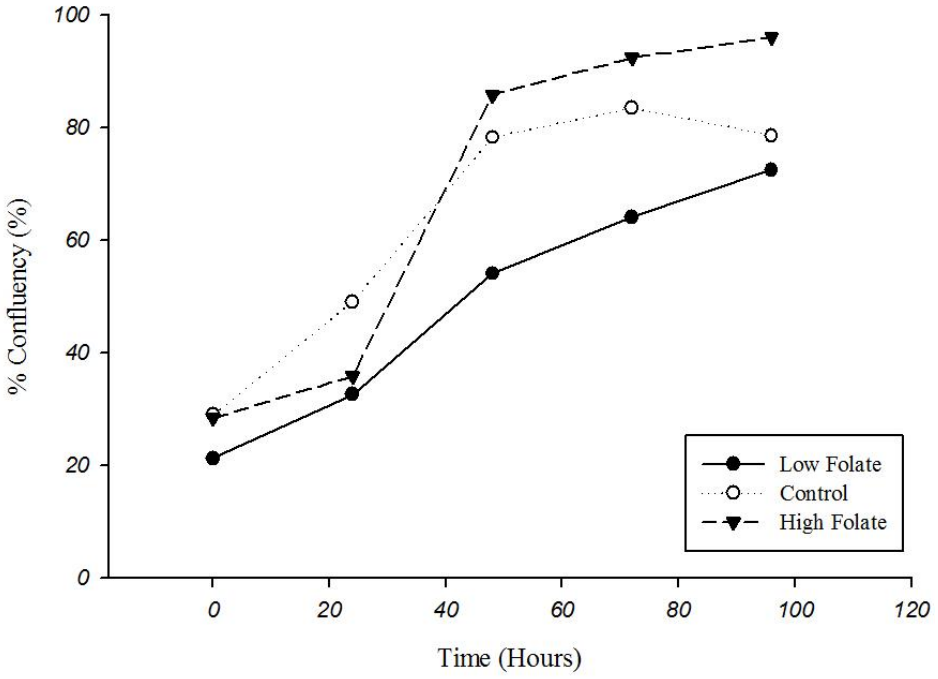


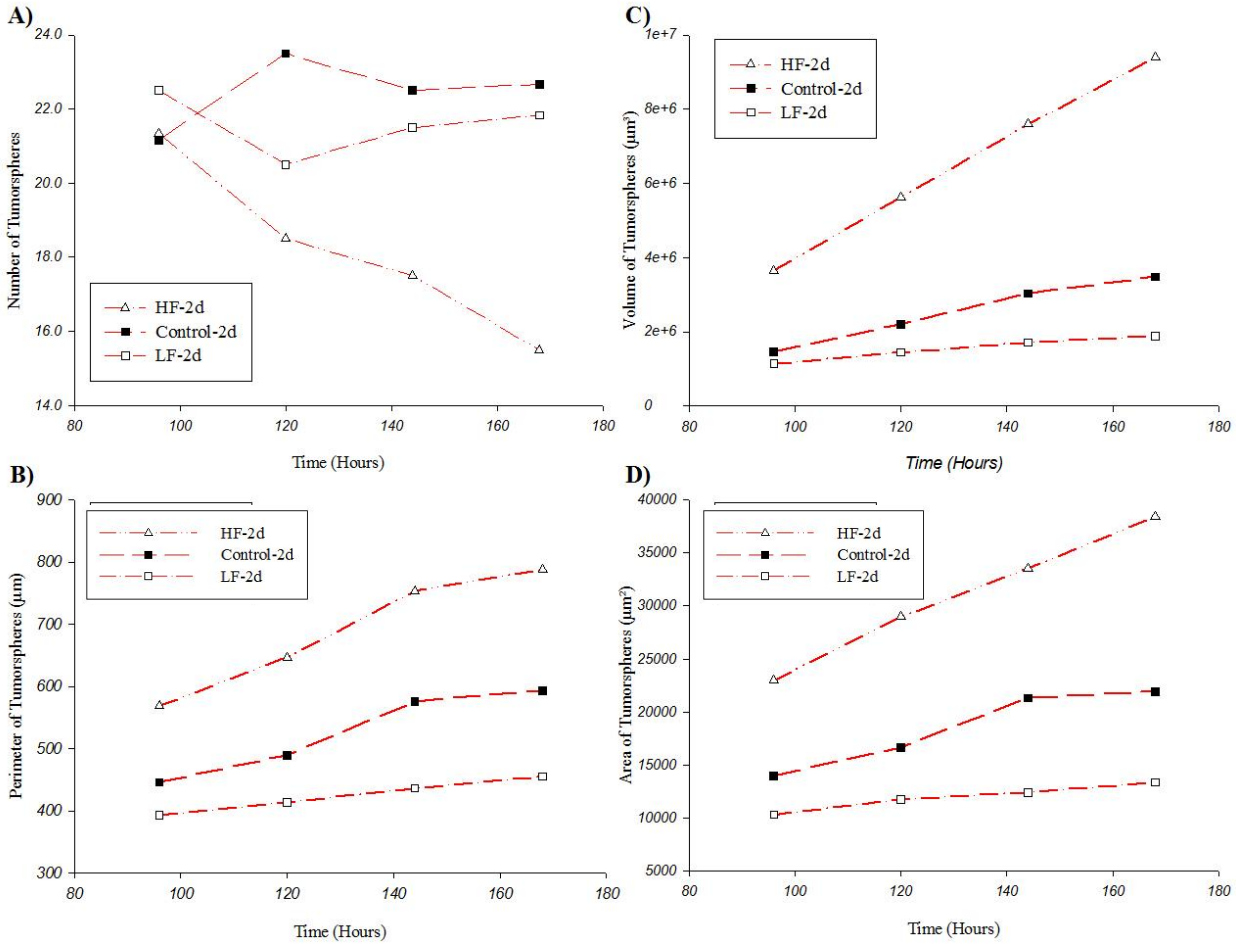
圖 1. MCF-7 細胞於不同處置的生長曲線。 MCF-7 細胞於在 log-growth phase 被培養於對照組，低葉酸和高葉酸的培養液連續四天。每天每個培養皿細胞的融合比例以 Celígo 計算。每個處置中細胞的倍增時間以 $\frac{Time_f - Time_i}{\log(\%confluence_f) - \log(\%confluence_i)} \times \log(2)$ 公式計算。低葉酸細胞平均倍增時間為 49.20 小時; 對照組細胞平均倍增時間為 35.49 小時; 而高葉酸組細胞平均倍增時間為 19.03 小時。

二、短期葉酸缺乏和葉酸補充於促進不依賴貼壁的腫瘤球體形成有明顯差異

腫瘤球體的數目在它們各自組別中於 4 天和 7 天之間沒改變（圖 2A）。當觀察腫瘤球體的體積時，可以看出在 4 天和 7 天之間於高葉酸處理的細胞生長的腫瘤球的體積有巨大的增加。然而，對於低葉酸和對照組，僅有輕微的增加（圖 2C）。這相似的型態可藉由面積（圖 2D）和周邊（圖 2B）的趨勢來顯示。這些趨勢可透過 Celígo 提供的圖像進一步證明（圖 2E）。細胞最初用其各組別的培養液處理 2 天，然後在含腫瘤球體培養液培養基中培養。在 3 天，6 天和 9 天拍攝圖像。在低葉酸和對照組中沒有顯著差異。然而，在高葉酸組中，腫瘤球體開始增加其大小。

三、長期葉酸缺乏和補充促進腫瘤球體形成

在腫瘤球體培養液培養 3 天後，低葉酸和高葉酸組於第 4 天的細胞看起來形成小規模的腫瘤塊，然而對照組仍保為單個細胞（圖 3A）。6 天後，所有三種組均具有體外腫瘤，對照組是最小和最少的，而高葉酸組是最大和最多的（圖 3A）。於腫瘤球體培養液培養 9 天可成功產生體外大腫瘤（圖 3A）; 可以觀察到 9 天培養的對照組腫瘤看起來類似於培養 6 天的高葉酸組。在各組培養 8 天後，再培養於腫瘤球體培養液後亦得到相似的型態。在 3 天，所有三組具有豐富的小腫瘤塊（圖 3B）。在第 6 天，低葉酸細胞形成大腫瘤塊，但是大小於 9 天時並沒有變大。（圖 3B）。對照組和高葉酸組，腫瘤塊於第 9 相較於第 6 天有顯著增長；唯一的區別是高葉酸組的腫瘤塊明顯大於對照組（圖 3B）。



E) 2-Day Treatment

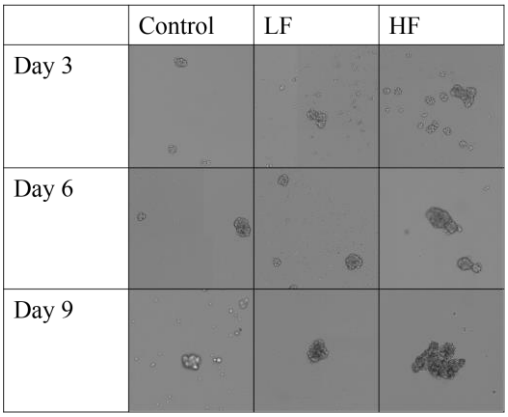


圖 2. 於 4 天和 7 天培養後以 Celigo 分析腫瘤球體。MCF-7 細胞在各組以其培養液處置 2 天及 11 天後，再以腫瘤球體培養液培養於 96 孔盤。 **(A)** 腫瘤球體的數目。**(B)** 平均周長。**(C)** 腫瘤球體平均估計體積，系統以:$\frac{\pi}{6} \times short\ diameter \times short\ diameter \times long\ diameter$公式計算。**(D)** 平均面積。**(E)** 腫瘤球成長照片。

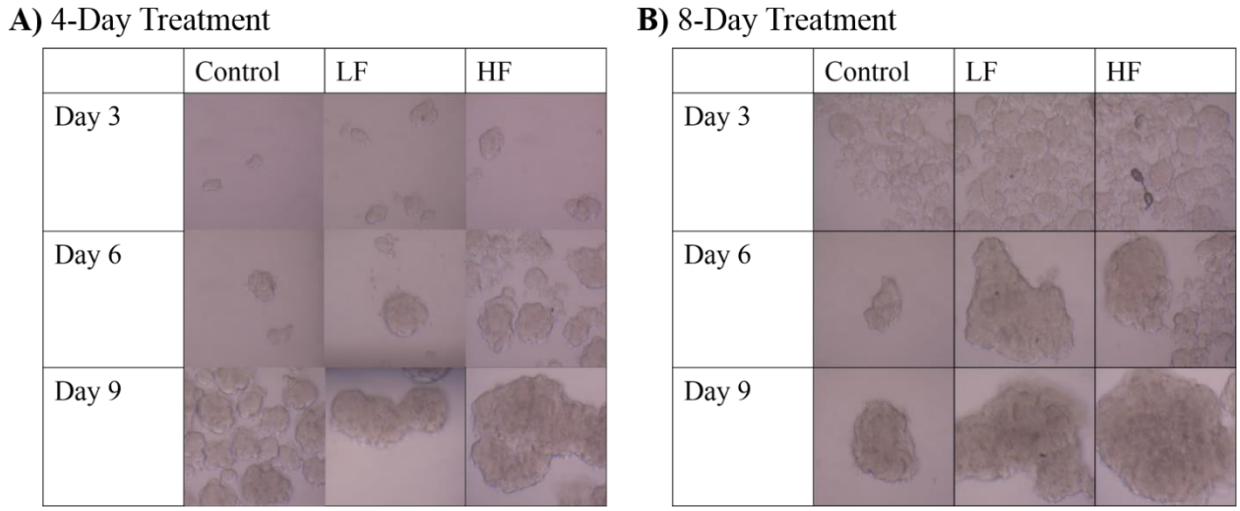


圖 3. MCF-7 細胞於不同組處置後腫瘤球體的成長。於每孔的腫瘤球體培養液中培養 5000 個經各組處置後的細胞，並於 3，6，9 天的培養後拍攝腫瘤的形成。**(A)** MCF-7 細胞於各組培養液處置細胞 4 天後– 對照組，低葉酸或高葉酸。**(B)** MCF-7 細胞於各組培養液處置細胞 8 天後– 對照組，低葉酸或高葉酸。

四、長期葉酸缺乏和補充促進癌幹細胞相關信號分子的表達

低葉酸和高葉酸組在 4 天處理後都比對照組表達更多的 *mTOR* 目標蛋白質;這種特徵在低葉酸和高葉酸兩組的 8 天處置後亦保持相同（圖 4A）。對於 4 天的處置後，p 值為 0.0005，而 8 天處置後的 p 值為 0.0193。在 4 天處置後，低葉酸組及高葉酸分別表達 4.8 倍及 2.2 倍高於對照組。在 8 天處置後，低葉酸組及高葉酸分別表達 7.1 倍及 1.2 倍高於對照組。對於 *SHH*，儘管兩組在 4 天處置後具有比對照組更多的蛋白質表達，但是高葉酸組在 8 天時表達低於對照組（圖 4B）。於 4 天處置後，p 值為 0.05，而 8 天處置後的 p 值小於 0.0001。在 4 天處置後，低葉酸組及高葉酸分別表達 1.41 倍及 3.451 倍高於對照組；在 8 天處置後，低葉酸組及高葉酸分別表達 3.93 倍及 0.84 倍相對於對照組。對於 *Sox-2*，低葉酸組具有低於對照組的初始表達，而高葉酸組具有更高的表達（圖 4C）。然而，這現象於 8 天處置的細胞切換，高葉酸組表達較少且低葉酸組表達比對照組多。於 4 天處置後的細胞，p 值為 0.1677，而 8 天處置後的細胞 p 值為 0.0066。在 4 天，相較於對照組低葉酸組表達 0.875 倍，高葉酸組表達 1.946 倍；在 8 天，低葉酸組表達 3.366 倍，而高葉酸組表達 0.21 倍。對於上述三種蛋白質，當將 4 天和 8 天處理與它們各自的培養基比較時，低葉酸對於所有三種蛋白質標誌物具有上調的表達，而高葉酸對於所有三種具有下調的表達。當測試 *Vimentin* 時，對於所有三種處理皆沒有表達。和 *Vimentin* 相反的是 *E-cadherin*，對於所有三種培養基，4 天和 8

天處理的表達水平保持相同（圖 4D）。對於 4 天處置的細胞，p 值為 0.1780，而 8 天處置的細胞 p 值為 0.7397。低葉酸組相對於對照組在 4 天處置的細胞表達 1.673 倍，在 8 天表達 1.342 倍。高葉酸細胞在 4 天表達 0.566 倍於對照組，在 8 天表達 0.9283 倍。值得注意的是，低葉酸表達大於對照組，而高葉酸表達較少。最後，對於 *Nanog*，處理 4 天的高葉酸細胞不表達任何 *Nanog* 蛋白；然而，在 8 天，它們開始表達一些，儘管仍然比對照組或低葉酸細胞的表達更低（圖 4E）。另一方面，低葉酸細胞的 *Nanog* 表達從 4 天到 8 天有降低的現象；且兩者均低於對照組（圖 4E）。高葉酸處理的細胞很少或沒有 *Nanog* 的表達，而低葉酸在 4 天及 8 天分別表達 0.9 及 0.7 倍於對照組。4 天處理的細胞，p 值為 0.0055；而 8 天處理的細胞 p 值為 0.1143。

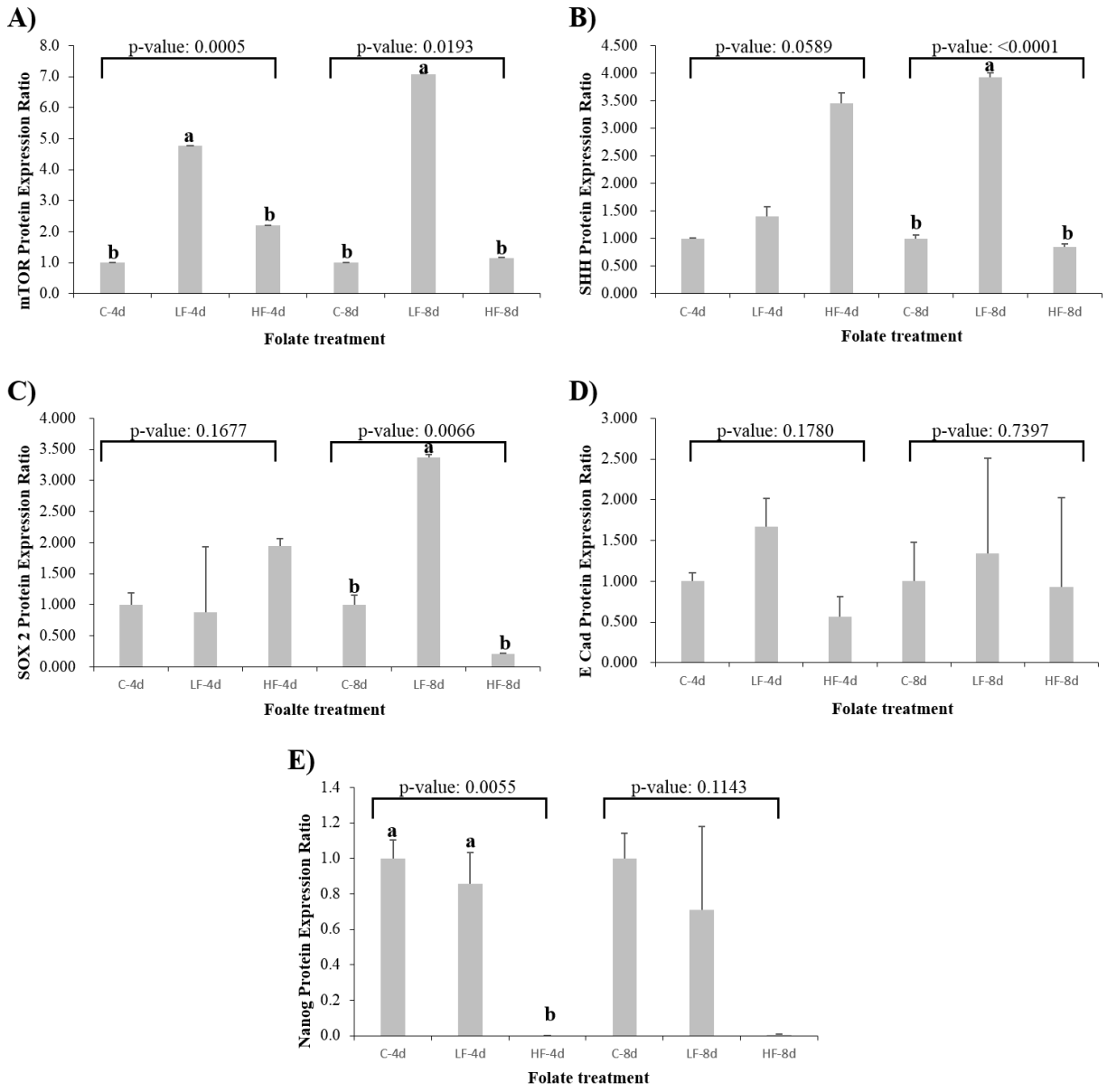


圖 4. *mTOR, SHH, Sox-2, E-Cadherin,* 和 *Nanog* 於 MCF-7 細胞的表達。 MCF-7 細胞分別於低葉酸，對照組及高葉酸的培養液處置 4 天和 8 天後，搜集細胞做各種蛋白質表達的試驗。利用 SAS9.4 軟體的單向 ANOVA 分析 p 值。用 Duncan's multiple-range test: 不同的上標當 P<0.05 時為明顯有差異。. **(A)***mTOR* 蛋白表現。**(B)***SHH* 蛋白表現。**(C)***Sox-2* 蛋白表現。**(D)***E-cadherin* 蛋白表現。**(E)***Nanog* 蛋白表現。

肆、討論

每種類型的培養基具有特定的葉酸濃度，其反映了已接受化療並補充葉酸的癌症患者血清中的平均葉酸濃度。Shahab-Ferdows 等人，建議在經過化療之病患有血清平均葉酸濃度 10 nM (Shahab-Ferdows *et al.*,2014)，而 Lamm 等人建議使用 HF 培養液含 50μM 葉酸的培養基 (Lamm *et al.*,2015)。

與對照組相比較，對於低葉酸處理的細胞有較低的倍增時間，這資料說明了微環境對葉酸缺乏的變化將導致乳腺癌細胞經歷細胞休眠 (圖 1)。在 2 天缺乏後，由 Celígo 得到的參數表明低葉酸處理的細胞形成比對照組更小和更小的腫瘤球，證明大多數 MCF-7 細胞僅在 2 天缺乏後不會轉化成癌幹細胞；微環境變化的影響並沒有明顯。然而，值得一提的是，一些細胞確實轉變成癌幹細胞，這可由於腫瘤球體培養液培養時形成一些腫瘤球得到證明 (圖 1)。在 4 天缺乏後，圖 2A 顯示了更多的低葉酸細胞已經轉變成癌幹細胞，並以比對照組更快的速度形成更大的腫瘤球。在 8 天缺乏後，甚至更多的腫瘤球形成於僅 3 天腫瘤球體培養液中的培養；因此，8 天缺乏後會導致更多的細胞轉變成癌幹細胞。腫瘤球的形成證實了癌幹細胞的自我更新特性。藉由幹細胞標記物的表達進一步說明了該趨勢。在 2 天、4 天和 8 天缺乏後引起腫瘤球生成的差異，暗示了缺乏的時間可以導致足以區別差異的想法。如 Murakami et al.發現，*mTOR* 是幹細胞生長和增生所必需的因子；使 *mTOR* 活性失去會損害其自身更新的能力 (Marukami *et al.*,2004)。對於 4 天和 8 天處理的低葉酸細胞具有比對照組更多的 *mTOR*，SHH 和 *Sox-2* 的表達，暗示更多的 MCF-7 細胞轉化成癌幹細胞並表達幹細胞的標記物。有趣的是，4 天和 8 天之間的模式表明細胞暴露於低葉酸的條件越長，更多的細胞將變為癌幹細胞並可能轉移。這與 Heiden 等人的發現相關，因為他們的結論是 *SHH* 途徑通過 Gli1 誘導的 *Snail* 表達維持癌幹細胞自我更新特性 (Heiden *et al.*,2014)。如 Pu Xia 和 Xia-Yan Xu 建議，*mTOR* 信號通路是癌症的主要調節因子：*mTOR* 複合物的激活增加信使核糖核酸的轉譯，蛋白質合成和細胞增生 (Xia and Xu, 2015)。

因為高葉酸細胞的倍增時間顯著低於對照組，對於高葉酸處理的細胞，數據顯示存在於微環境中的葉酸增加會提高細胞增殖的速度。在 2 天補充後，Celígo 提供的參數表明許多 MCF-7 細胞已經轉變成癌幹細胞，因為腫瘤球的體積，面積和周長以最

快的速度增加。圖 3A 顯示在腫瘤球體培養液中培養 4 天和 7 天之間存在的腫瘤球的計數的下降。這是因為許多腫瘤球團聚在一起並形成更大的腫瘤球，這可由在該期間球體尺寸的顯著增加來證明。這種類似的趨勢可用細胞補充 4 天高葉酸來增強。雖然在 3 天，高葉酸腫瘤球體具有和低葉酸細胞類似的大小和數量；從 6 天開始，高葉酸腫瘤球的數量和大小就存在顯著的差異。腫瘤球的快速生長證實了以下觀點：更多的高葉酸細胞通過其自我更新特徵轉移成癌幹細胞。這種模式可通過細胞補充 8 天高葉酸再次看見。這與 Manshadi 等人的結果相關:她的結論是，葉酸補充顯著促進了乳腺腫瘤的進展，並與控制飲食組相比有更高的腫瘤重量，體積和面積 (Manshadi *et al.*, 2014)。此外，高葉酸細胞在 4 天處理後具有 *mTOR*，*SHH* 和 *Sox-2* 的過度表達；該表達在 8 天處理時降低，揭示那些幹細胞標記物的表達在 4 天中達到峰值，並同時將向下游方向激活信號。

對於 *SHH* 和 *Sox-2* 表達，僅 8 天處理的細胞呈現顯著差異，揭示更長的細胞暴露於微環境的變化，無論是低葉酸還是高葉酸，變化對癌細胞的影響越大。在各種處置中 *E-cadherin* 表達的差異證明是不顯著的（圖 4D）。一個潛在的推理是因為 *E-cadherin* 不是 MCF-7 的主要上皮標誌物。 Zhang 等人以 4 種類型的標記：*E-cadherin, β-cadherin, N-cadherin* 和 *Vimentin* 測試了 MCF-7 細胞的 EMT。在四個之中，*β-cadherin, N-cadherin* 和 *Vimentin* 在處置之間存在顯著差異，而 *E-cadherin* 表達水平輕微下降 (Zhang *et al.*, 2013)。由於兩種處置不調節 MCF-7 細胞的 *Nanog* 過杜表達的事實（圖 4E），數據表明葉酸缺乏和葉酸補充可以表觀遺傳地重編幹細胞基因標籤，例如 *Nanog* 標記，而不是被 Hedgehog 信號傳導激活 (Chatel *et al.*, 2007)。

這些發現強化了實驗數據的相關性，表明在癌發生期間葉酸的時間和劑量是扮演重要的角色。 Ulrich 得出結論，在腫瘤形成之前葉酸增加可以防止腫瘤發展，但是在病灶存在後補充合成的葉酸卻會增強腫瘤的進展 (Ulrich, 2007)。

有了所有這些資料，實驗仍存在一些限制。通過與癌幹細胞相關標記的特定基因表達來分析腫瘤與葉酸間的相互作用仍可能不反映人類乳腺癌組織中的真實關係。然而，由於腫瘤標本的不易取得，我們需要進一步的研究來確認彼此的相互關係。此外，每個患者的葉酸水平可能不同，這取決於他們的化療和葉酸補充；因此，本研究中使用的培養基的葉酸濃度是使用一般群體的粗略估計值。雖然 *SHH* 和 *mTOR* 信號通

路似乎控制乳腺癌細胞，從而控制癌幹細胞，在這項研究中，體外環境不能模仿複雜的人類系統。前瞻性設計研究分析人體中的途徑是有必要的。

伍、結論

體外數據精確地指出，低葉酸和高葉酸的微環境促進體外腫瘤形成和轉變為癌幹細胞，這將增加乳腺癌細胞轉移的機會。短期暴露於葉酸缺乏，雖然轉變的細胞的百分比較低，但仍會促進向癌幹細胞的轉變；另一方面，長期暴露會明顯地誘導癌細胞轉成癌幹細胞，表明低葉酸微環境可以增加轉移率。相同地，藉由幹細胞標記的表達和腫瘤球形成的顯著增加所示，補充葉酸的短期和長期治療都可導致 *MCF-7* 細胞轉化為癌幹細胞。這兩個指示性的發現表明，因為化學療法和葉酸補充以抵消葉酸的不平衡將使治療藥物的治療失效，故我們需要對乳腺癌患者進行新的治療研究。

需要進一步的研究來研究如何調節從癌細胞向癌幹細胞的轉變以及與兩種微環境相關的具體機制。此外，有必要通過將體外腫瘤球注射到動物體內以觀察是否發生轉移來測試體內結論。通過適當的檢驗，乳腺癌患者的新型治療策略可根據其營養狀況個性化，以通過優化治療時間來最小化癌症轉移發生率。

陸、參考文獻

(1) Jin X and Mu P. “Targeting Breast Cancer Metastasis.” *Libertas Academica* 9.1 (2015): 23-24.

(2) Sharma G, Dave R, Sandaya J, Sharma P, Sharma K. “Various Types and Management of Breast Cancer: An Overview.” *Journal of Advanced Pharmaceutical Technology and Research* 1.2 (2010): 109-126.

(3) Liotta LA and Kohn EC. “The Microenvironment of the Tumor-host Interface.” *Nature* 411.6825 (2001): 375-379.

(4) Lapidot T, Sirard C, Varmoor J, Murdoch B, Hoang T, Caceres-Cortes J *et al.* “A Cell Initiating Human Acute Myeloid Leukaemia after Transplantation into SCID Mice.” *Nature* 367.6464 (1994): 645-648.

(5) Cukierman E and Bassi DE. “The Mesenchymal Tumor Microenvironment: a Drug-resistant Niche.” *Cell Adhesion and Migration* 6.3 (2012): 285-296.

(6) Curtin J and Lorenzi M. “Drug Discovery Approaches to Target Wnt Signaling in Cancer Stem Cells.” *Oncotarget* 1.7 (2010): 552-566.

(7) Maenhaut C, Dumont JE, Roger PP, and Van Staveren WCG. “Cancer Stem Cells: a Reality, a Myth, a Fuzzy Concept or a Misnomer? An Analysis.” *Carcinogenesis* 31.2 (2010): 149-158.

(8) Cao L, Zhou Y, Zhai B, Liao J, Xu W, Zhang R *et al*. “Sphere-forming Cell Subpopulations with Cancer Stem Cell Properties in Human Hepatoma Cell Lines.” *BioMed Central Gastroenterology*, 11, 71, 2011.

(9) Velasco-Velázquez MA, Popov VM, Lisanti MP, Pestell RG. “The Role of Breast Cancer Stem Cells in Metastasis and Therapeutic Implications.” *The American Journal of Pathology,* 179, 1, 2011, 2-11.

(10) Tang DG. “Understanding Cancer Stem Cell Heterogeneity and Plasticity.” *Nature Cell Research* 22 (2012): 457-472.

(11) Shen CN, Burke ZD, and Tosh D. “Transdifferentiation, Metaplasia and Tissue Regeneration.” *Organogenesis* 1. 2 (2004): 36-44.

(12) Puisieux A, Barbletz T, and Caramel J. “Oncogenic Roles of EMT-inducing Transcription Factors.” *Nature Cell Biology* 16 (2014): 488-494.

(13) Mukherjee S, Frolova N, Sadlonova A, Novak Z, Steg A, Page GP *et al.* “Hedgehog Signaling and Response to Cyclopamine Differ in Epithelial and Stromal Cells in Benign Breast and Breast Cancer.” *Cancer Biology and Therapy* 5.6 (2006): 674-683.

(14) Hadjimichael C, Chanoumidou K, Papadopoulou N, Arampatzi P, Papamatheakis J, and Kretsovali A. “Common Stemness Regulators of Embryonic and Cancer Stem Cells.” *World Journal of Stem Cells* 7.9 (2015): 1150-1184.

(15) Xia P and Xu XY. “PI2K/Akt/mTOR Signaling Pathway in Cancer Stem Cells: from Basic Research to Clinical Application.” *American Journal of Cancer Research* 5.5 (2015): 1602-1609.

(16) Chiarugi A, Dolle C, Felici R, and Ziegler M. “The NAD Metabolome – a Key Determinant of Cancer Cell Biology.” *Nature Reviews Cancer* 12.11 (2012): 741-752.

(17) Sun M, Yang C, Zheng J, Wang M, Chen M, Le DQS *et al.* “Enhanced Efficacy of Chemotherapy for Breast Cancer Stem Cells by Simultaneous Suppression of Multidrug Resistance and Antiapoptotic Cellular Defense.” *Acta Biomaterialia* 28 (2015): 171-182.

(18) Eyler CE and Rich JN. “Survival of the Fittest: Cancer Stem Cells in Therapeutic Resistance and Angiogenesis.” *Journal of Clinical Oncology* 26.17 (2008): 2839-2845.

(19) *Stover PJ*. “One-Carbon Metabolism-Genome Interactions in Folate-Associated Pathologies.” *The Journal of Nutrition* 139.12 (2009): 2402-2405.

(20) Zhao R, Matherly LH, and Goldman ID. “Membrane Transporters and Folate Homeostasis; Intestinal Absorption Transport into Systemic Compartments and Tissues.” *Expert Reviews in Molecular Medicine Journal* 11.4 (2009).

(21) Visentin M, Zhao R, and Goldman ID. “The Antifolates.” *Hematology/Oncology Clinics of North America* 26.3 (2013): 629-632.

(22) Stockstad, E.L.R. “Historical Perspectives on Key Advances in the Biochemistry and Physiology of Folates.” *Current Issues in Clinical Nutrition* 13 (1990).

(23) Wien TN, Pike E, Wisloff T, Staff A, Sigbjorn S, and Klemp M. “Cancer Risk with Folic Acid Supplements: a Systematic Review and Meta-analysis.” *BMJ Open* 2.1 (2012).

(24) Yi K. “Folate and Colorectal Cancer: an Evidence-based Critical Review.” *Molecular Nutrition and Food Research* 51.3 (2007): 267-292.

(25) Shahab-Ferdows S, Engle-Stone R, Hampel D, Ndjebayi A, Nankap M, Brown K *et al.* “Risk Factors for Folate Deficiency Differ from Those for Vitamin B12 Deficiency in Cameroonian Women and Children.” *The FASEB Journal* 28.1 (2014).

(26) Lamm N, Maoz K, Bester AC, Im MM, Shewach DS, Karni R *et al.* “Folate Levels Modulate Oncogene-induced Replication Stress and Tumorigenicity.” *EMBO Molecular Medicine* 7.9 (2015): 1138-1152.

(27) Strickland KC, Krupenko NI, and Krupenko SA. “Molecular Mechanisms Underlying the Potentially Adverse Effects of Folate.” *Clinical Chemistry and Laboratory Medicine* 51.3 (2013).

(28) Murakami M, Ichisaka T, Maeda M, Oshiro N, Hara K, Kiyama H et al. “mTOR Is Essential for Growth and Proliferation in Early Mouse Embryos and Embryonic Stem Cells” *Molecular and Cellular Biology* 25.12 (2004): 6710-6718.

(29) Heiden KB, Williamson AJ, Doscas ME, Ye J, Wang Y, Liu D *et al.* “The Sonic Hedgehog Signaling Pathway Maintains the Cancer Stem Cell Self-Renewal of Anaplastic Thyroid Cancer by Inducing Snail Expression.” *The Journal of Clinical Endocrinology and Metabolism* 99.11 (2014).

(30) Manshadi SD, Ishiguro L, Sohn KJ, Medline A, Renlund R, Croxford R *et al*. “Folic Acid Supplementation Promotes Mammary Tumor Progression in a Rat Model.” *PLoS One* 9.1 (2014).

(31) Zhang X, Li Y, Zhang Y, Song J, Wang Q, Zheng L *et al.* “Beta-elemene blocks epithelial-mesenchymal transition in human breast cancer cell line MCF-7 through Smad3-mediated down-relation of nuclear transcription factors.” *PLoS One* 8.3 (2013).

(32) Chatel G, Ganeff C, Boussif N, Delacroix L, Briquet A, Nolens G *et al.* “Hedgehog Signaling Pathway is Inactive in Colorectal Cancer Cells Lines.” *International Journal of Cancer* 121.12 (2007): 2622-2627.

(33) Ulrich CM. “Folate and Cancer Prevention: a Closer Look at a Complex Picture.” *The American Journal of Clinical Nutrition* 86.2 (2007): 271-273.

Introduction

Breast cancer causes more than 0.5 million deaths every year; over 90% of patients die of metastasis, which is the formation of tumors at a distance from the primary site [1]. The metastasis lesions damage vital organs, often lymph nodes, lung, liver, bone, brain, thyroid, and the heart. The median survival rate of females with metastatic cancer is merely two to three years [1]. The heterogeneous nature of breast cancer makes it hard to find the cure and asses the risk factors for metastasis [2]. Although established risk factors of breast cancer include significant family history, alteration in hormonal level, sedentary life style, high dietary intake of fat, and environment, the molecular pathways to breast cancers' metastasis are unclear [2].

The cancer stem cell (CSC) theory has been proposed by Lapidot *et al.* in the early 1990s to explain the origin of neoplasms [3]. Lapidot identified that only a small percentage of leukemic cancer cells could cause cancer because of neoplastic stem cells [4]. Similar results for different types of neoplasms support that the low population of CSCs causes cancer growth and chemoresistance [4]. According to the CSC theory, CSCs exist in a similar manner as other stem cells, existing in niches with suitable microenvironment of low pO_2 and remaining in the G0 phase of cell cycle for longer periods due to their longevity [4]. This makes it easier for CSCs to mutate and alter genetically/epigenetically [4]. The niche sends signals directing CSCs to undergo self-renewal in order to maintain a subpopulation and differentiate to generate tumor bulk [5]. Maenhaut *et al.* pinpointed the three main characteristics of CSCs: (1) self-renewal; (2) trans-differentiation; and (3) expression of stemness markers [6]. Stem cells with self-renewal capabilities are rare as the basic requirements are having niches with both supporting (epithelial or stromal) cells, extracellular matrix, blood vessels, and nerve fibers, causing most stem cells to remain dormant with the proliferative potential [7].

Self-renewal of a cell is characterized by the formation of anchorage-independent tumorspheres [8]. Velasco-Velazquez *et al.* found that when breast cancer cells transition into CSCs, they possess the capacity to divide asymmetrically and produce stem cells that can self-renew and progenitor cells that can generate heterogeneous lineages of the cancer cells with tumors [9]. The ability to form tumors serves as an evidence of metastasis [9].

Trans-differentiation describes the conversion of one cell type to another [10]. For CSCs specifically, they undergo a reversible epithelial to mesenchymal transition (EMT), in which polarized epithelial cells convert into motile mesenchymal ones [11]. EMT is

dependent on the microenvironmental signals regulated by the EMT-inducing transcription factors (EMT-TFs) that control the expression of proteins involved in cell-cell contact, cell polarity, cytoskeleton structure, and extracellular matrix degradation [12]. During the process, epithelial genes are downregulated and replaced by mesenchymal genes [12]. Key epithelial biomarkers include *E-cadherin*, *cytokeratin*, *occluding*, and *ZO-I*; mesenchymal markers include *matrix metalloproteinases* (*MMPs*), *N-cadherin*, *fibronectin*, and *vimentin*. Among all, the loss of *E-cadherin* expression, direct or indirect, is the most crucial step in EMT.

Sonic hedgehog (*SHH*) signaling regulates the interactions of EMT in proliferation and differentiation of cells [12, 27]. EMT-TFs can alleviate a cell's oncosuppressive mechanisms that maintain epithelial traits, causing cells to be malignant and develop tumors. CSCs from epithelial tissues exhibit embryonic stem cell (EMC)-like biomarkers that incorporates oncogene *c-Myc* and pluripotency factors like *Sox-2* and *Nanog* [13]. The *mTOR* pathway is activated during various cellular processes, most notably, tumor formation and is considered as a master regulator for cancer. *mTOR* signaling maintains the self-renewal and tumorigenicity of CSCs [14, 15]. The increase in CSCs, hence, will induce *SHH* and *mTOR* singling and the expression of *Sox-2* and *Nanog*, suggesting the formation of tumorspheres that can express CSCs phenotypes and cause metastasis of breast cancer.

Chemotherapy has been recognized on a world-wide scale to be a frontline treatment strategy for breast cancer. Cancer cell's squired multidrug resistance causes chemotherapy to not have a guaranteed success [16].The drug resistance capability of cancer cells stems from CSCs in malignant tumors [16]. The popular and common approach of chemotherapy and radiotherapy diminishes those rapidly growing cancer cells of a tumor mass; it does not prevent recurrence, demonstrating its failure at removing CSCs [17]. Instead, many suggest that these therapies enrich the CSCs [17]. Often, advanced cancers recur after treatments that initially led to therapeutic responses [17].

For treatment of breast cancer, patients are often prescribed with chemotherapeutic, antifolate drugs [18]. These drugs limit the production of folic acid, which is essential to some folate-dependent enzymes and their role in cellular production process, specifically, the one-carbon metabolism [19]. One-carbon metabolism mediates the *de novo* DNA synthesis cycle as it synthesizes purines and thymidylates, and remethylazes homocysteine to methionine, an essential amino acid for protein synthesis [19]. Folate acts as a co-enzyme in one-carbon metabolism; yet, folate cofactors are the limiting factors in the metabolic cycle as

the concentration of folate-dependent enzymes and folate-binding proteins far exceeds that of folate cofactors [19].

Chemotherapy causes folate deficiency (FD), creating low folate metabolic stress (LFMS) [20]. LFMS is identified as the epigenetic modifier and mutagenic factor that promotes carcinogenesis [21]. To counteract FD, folic acid (FA) supplements are prescribed to balance the folate status and prevent adverse events associated with antifolate medication [22]. Recently, there have been rising concerns over FA supplementation because it might increase the risk of cancer as they promote progression of preneoplastic and undiagnosed neoplastic lesions [23]. FA could also boost cancer cells' folate metabolism and promote proliferation, first observed by Sidney Farber in 1940s [24]. However, the controversy of FA supplementation and cancer still lingers up till today. Hence, it is crucial to identify the molecular mechanisms in which FD or FA supplementation impact proliferation and tumorigenesis. This study seeks to shed light on how low folate (LF) and high folate (HF) microenvironments in cancer patients can potentially affect the breast cancer cells' transition into CSCs and their tumorigenesis ability.

Method and Materials

Cell Line Culture

The MCF-7 (human breast adenocarcinoma) cell line used in this study was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured 100 mm diameter cell culture dishes in Roswell Park Memorial Institute (RPMI) 1640 (Gibco Lab, Grand Island, NY), with 10% fetal bovine serum (FBS, Gibco Lab, Grand Island, NY), 100 IU/ml streptomycin (Streptomycin sulphate, Sigma, St. Louis, MO), 100 IU/ml penicillin (Pronapen, Sigma, St. Louis, MO), and 0.2% sodium bicarbonate ($NaHCO_3$, Sigma, St. Louis MO). The pH of the medium was adjusted to 7.4 with 1N HCl. Control medium has a folate concentration of 2.2 µM. This is known as the control medium for the purpose of this research. The cells were incubated in 37°C with 5% carbon dioxide (CO_2).

In order to have folate deficiency or FA supplementation, the MCF-7 cells that were originally grown in the control medium were treated with LF and HF medium. The LF medium consists of RPMI 1640 without folic acid (Gibco Lab, Grand Island, NY), 10% FBS, 100 IU/ml streptomycin, 100 IU/ml penicillin, and 10 nM folic acid. The final pH of 7.4 was calibrated using 1N HCl. The HF medium is the exact same thing as the LF medium, with the exception that it includes 50µM folic acid.

Confluency Assay by Celígo

MCF-7 cells treated for four days using the three different mediums were used to observe cell growth. 2×10^5 cells were seeded into each well of the 96 wells plate (Corning, Corning NY) for three repeats for each treatment. Plates were scanned by Celígo (Nexcelom, Lawrence, MA) once a day over four-day period to test for the cells' confluency.

Tumorspheroid Assay by Celígo

MCF-7 cells treated for 2 days were subjected to Celígo's tumorspheroid assay. 3×10^4 cells were seeded into each well of the 6 well ultra-low attachment plates. The spheroids were cultured in the oncospheroid medium for 7 days. Oncospheroid medium consists of Dulbecco's Modified Eagle Medium-F12 (DMEM-F12, Gibco Lab, Grand Island, NY) with 2% 50X B-27 Supplement (Thermo, Rockford, IL), 10ng/ml Recombinant Human Fibroblast Growth Factor (rhFGF, Sigma, St. Louis, MO), 1% streptomycin, 1% penicillin, and 20 ng/ml Recombinant Human Epidermal Growth Factor (rhEGF, Sigma, St. Louis, MO). Starting from day 4, the plates were scanned by Celígo, which automatically generates data of the following parameters: (1) tumorsphere count; (2) estimated volume (μm^3); (3) area (μm^2); and (4) perimeter (µm).

Oncospheroid Assay Cultivation

After 4d and 8d treatment in the LF, control, and HF medium, MCF-7 cells were reseeded into 6-well ultra-low attachment plates (Corning, Corning, NY) at a density of 5000 cells/well using the oncospheroid medium. At 3, 6, and 9 days intervals, pictures of the tumorspheroids were taken and the number of tumorspheroids in each well was counted in order to track the growth of the tumorspheroids.

Western Blot

In addition to oncospheroid assay, the MCF-7 cells treated in the three respective mediums after 4 and 8 days were collected in order to conduct western blot. Cells were first washed with 1x phosphate-buffered saline (PBS, Sigma, St. Louis, MO) twice and then lysed with 50ul of 1X lysis buffer (10x lysis buffer (Cell Signaling Technology, Boston, MA), purified water, 10% 100x Protease inhibitor (PI, Thermo Fisher), and 1% phenylmethylsulfonyl fluoride (PMSF, Thermo, Rockford, IL). After the cells were collected into Eppendorf, they were placed on ice for 15 minutes, preceding centrifugation for 30 minutes in 4^{o}C at 13200 rpm. Proteins in the supernatant were collected and stored in -20^{o}C for future use.

Protein concentrations were determined using the BSA protein assay kit (Bio-Rad, Hercules, CA) in order to standardize the amount of protein loaded in western blots. 5x Bio Red protein assay (Bio-Rad, Hercules, CA) was diluted to 1x using deionized water. BSA standard was diluted into the following concentrations to create standards: 12.5 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 375 µg/ml, and 500 µg/ml. 10 µl of each standard was loaded into a well with 200 µl 1x Bio Red protein assay. After 5 minutes, the optical densities (OD) were tested using the Elisa Reader (Dynatech Laboratories, West Sussex, UK) at 595 nm. With a R^2 greater than 0.99, the samples' protein concentrations were tested. 10 µl of each sample was loaded into a well with 200 µl of 1x Bio Red protein assay; then, using Elisa reader, at 595 nm, the OD values were tested. With the given protein concentration, stocks of 40 µg/ul of protein were prepared by adding 3µl 6x dye (Thermo, Rockford, IL) and deionized water to reach a total of 18 µl. The stocks were centrifuged, heated at 105^{o}C for 10 minutes, and placed on ice for 10 minutes.

Proteins were resolved on 8% sodium dodecyl sulfate (SDS) gels and transferred to polyvinylidene difluoride (PVDF, Thermo, Rockford, IL) membranes for 45 minutes. Membranes were cut in accordance to the marker and the molecular weight of the antibodies. They were blocked for two 30 minutes intervals in 5% nonfat dry milk in TBST (Sigma, St. Louis, MO) at room temperature. After washing with TBST for 3 times, each is 5 minutes, the membranes probed for 18 hours with primary antibodies diluted in TBST as per the supplier's recommendations. Membranes were then washed with TBST for 5 minutes for 3 times and incubated for 1 h with secondary

antibodies in 5% nonfat dry milk (Anchor, Taipei, Taiwan). SuperSignal West Femto Chemiluminescent Substrate (Thermo, Rockford, IL) was added onto each membrane, which was then visualized by the UVP BioSpectrum Imaging System (Upland, CA).

The protein expression tested for this research are *Actin* (Santa Cruz Biotechnology, Santa Cruz, CA), *SHH* (Santa Cruz Biotechnology, Santa Cruz, CA), *Sox-2* (Genetex, Irvine, CA), *Nanog* (Genetex, Irvine, CA), *E-caderin* (BD Biosciences, Franklin Lakes, NJ), *Vimentin* (Cell Signaling Technology, Boston, MA), and *mTOR* (Genetex, Irvine, CA). *Actin* served as the positive control and was diluted at a ratio of 1:20000. *SHH* and *Sox-2* were diluted at a ratio of 1:1000, *Nanog* at 1:1500, *mTOR* at 1:1000, and E-cad and Vimentin at 1:10000. For secondary antibody staining, *mTOR*, *SHH*, *Sox-2*, *Nanog*, and *Vimentin* were stained with anti-Rabbit *IgG* (Genetex, Irvine, CA) at a dilution factor of 1:2000. Actin was stained with goat anti-mouse *IgG* (Genetex, Irvine, CA) at a ratio of 1:20000. *E-caderin* was stained with goat anti-mouse *IgG*, but at a ratio of 1:2000.

Statistical Analysis

This research uses SAS 9.4 software (SAS Institute, Inc., Cary, NC) to run statistical analysis. Data were subjected to a one-way analysis of variance (ANOVA) and followed by Duncan's multiple range test to determine differences by treatments and durations. Results were considered statistically significant at $P<0.05$.

Results

LF and HF cultivations modulate growth of MCF-7 cells. Cultivation of MCF-7 cells in the control medium reached 80% confluency prior to 72h (Fig. 1), with a steady increase from 0h. Though after 72h, confluency level began to drop (Fig. 1), suggesting that cells have detached from the cell culture plate and died. Without considering the plateaued cell growth, MCF-7 cells in the control medium has an average doubling time of around 35.49h. Compared to the control, those grown in LF medium did not reach 80% confluency until after 96h (Fig. 1); yet, the growth rate started to plateau after 48h. On average, cells in LF medium have a doubling time of 49.20h. On the contrary, the MCF-7 cells treated with HF medium reached 80% confluency within 48h, followed by a steady slow to no growth phase until 96h (Fig. 1). These cells have an average doubling time of 19.04h.

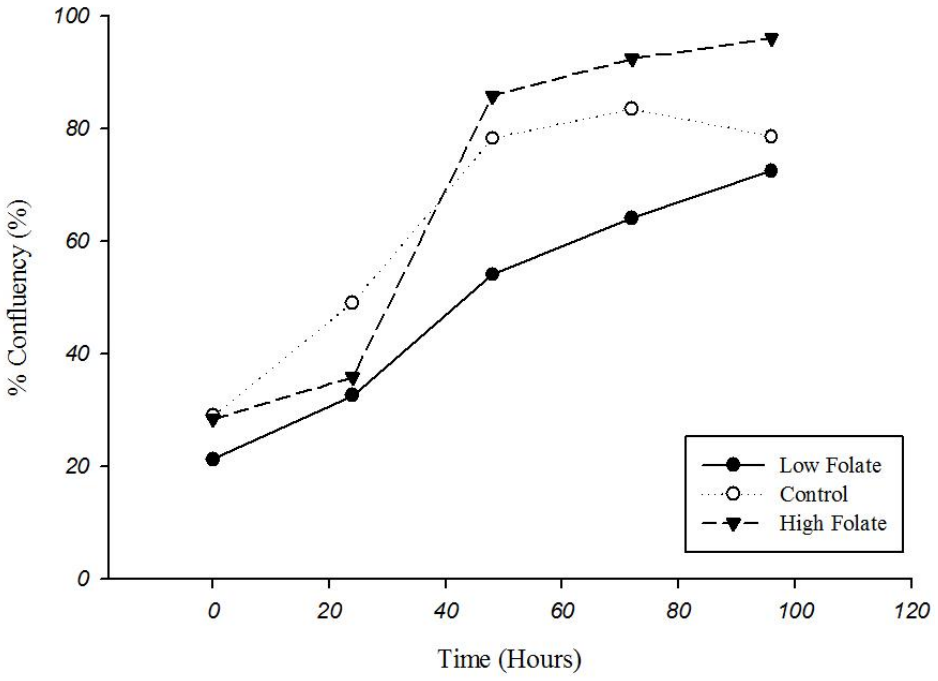


Fig 1. MCF-7 cells growth curve under different folate treatments. MCF-7 cells at log-growth phase were cultured with control, LF, and HF medium for 4 continuous days. Each day, the percent confluency of each plate was provided by Celígo. The doubling time of the cells under each treatment is calculated using the formula $\frac{Time_f - Time_i}{\log(\%confluence_f) - \log(\%confluence_i)} \times \log(2)$. LF cells have an average doubling time of 49.20h; control cells have an average of 35.49h; and HF cells' doubling time is around 19.04h.

Anchorage-independent tumorspheroid formation is differentially affected by short-term folate depletion and folate supplementation. The tumorsphere count does not change between 4d and 7d for their respective groups (Fig. 2A). When observing the volume of tumorspheres, one can see that there is a huge increase in the volume of the tumorspheres grown from HF-treated cells between 4d and 7d. For the LF and control groups, however, there is only a slight increase (Fig. 2C). This similar pattern is expressed through the trend of the area (Fig. 2D) and the perimeter (Fig. 2B). These trends are further justified through the images provided by Celígo (Fig. 2E). The cells were initially treated with their medium for 2 days prior to their cultivation in the oncospheroid medium. Images were taken at 3d, 6d, and 9d. There is no significant difference in the LF and control groups. However, in the HF group, the tumorspheres increased in size.

Long-term folate depletion and supplementation promote tumorspheroid formation. After 3d treatment in the oncospheroid medium, the 4d treated cells of LF and HF appear to form small-scaled tumor masses, while that of control remain as a single cell (Fig. 3A). After 6d, all three types had *in vitro* tumors, with control being the smallest and least abundant ones and HF being the largest and most abundant ones (Fig. 3A). 9d treatment in oncospheroid medium successfully generated large tumors *in vitro* (Fig. 3A); it can be observed that the control treatment at 9d looks similar to the HF treatment at 6d. Similar pattern was observed when culturing the 8d treated cells in oncospheroid medium. At 3d, all three types have abundant small tumor masses (Fig. 3B), compared to the ones formed when the cells were treated for 4d under their respective mediums. At 6d, LF cells formed large tumor masses, yet did not grow in size between then and 9d (Fig. 3B). For both control and HF, the tumor masses grew significantly between 6d and 9d; the only difference is that HF tumor masses were drastically larger than those of the control treatment (Fig. 3B).

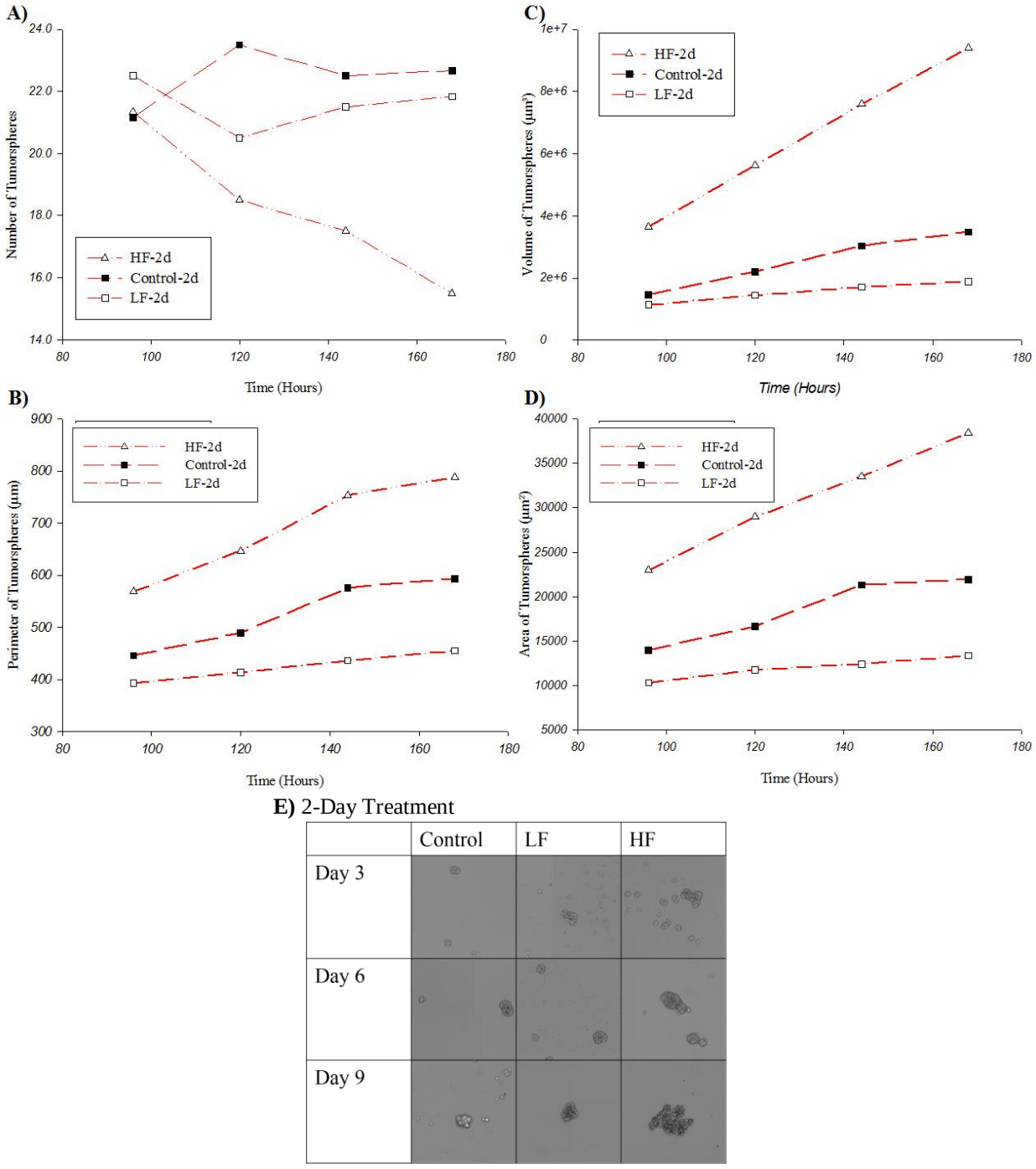


Fig 2. Celígo analysis of tumorspheres between 4d and 7d cultivation. MCF-7 cells were cultivated in oncospheroid mediums in 96-well plates after 2d and 11d treatments in their respective mediums. **(A)** Tumorsphere Count. **(B)** Perimeter of tumorspheres. **(C)** Volumes of tumorspheres, calculated by the system using the following algorithm: $\frac{\pi}{6} \times short\ diameter \times short\ diameter \times long\ diameter$. **(D)** Area of tumorspheres. **(E)** Image of tumorspheres at different times periods under different folate treatments.

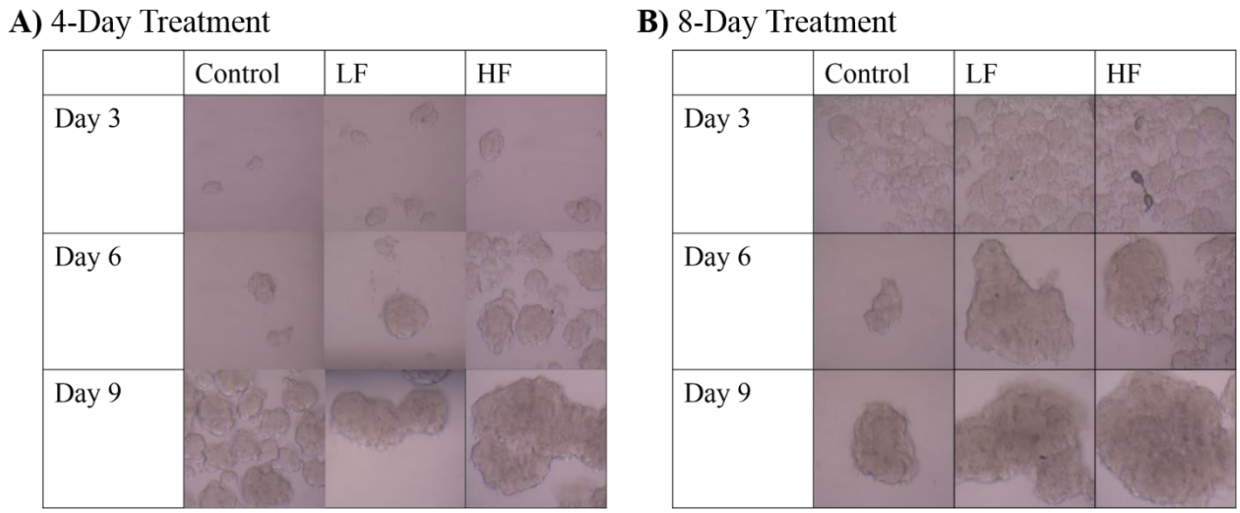


Fig 3. Oncospheroid Tumors Grown from MCF-7 Cells Treated with Different Mediums. 5000 cells in each well were cultured in oncospheroid medium after their respective treatment times and images of the tumor formation were taken at days 3, 6, and 9 during the cultivation. (**A)** MCF-7 cells were treated for 4 days in their respective mediums – either control, LF, or HF. (**B)** MCF-7 cells were treated for 8 days in their respective mediums.

Long-term folate depletion and supplementation promote expressions of CSC-associated signaling molecules. For *mTOR* target protein, both LF and HF at 4d treatment express more than the control group; this characteristics remains the same at 8d treatment for both LF and HF (Fig. 4A). For 4d treatment group, the p-value is 0.0005, while that of the 8d treatment group is 0.0193. At 4d, LF expresses 4.8 fold and HF expresses 2.2 fold more than control. At 8d, LF expresses 7.1 fold and HF expresses 1.2 fold more than control. For *SHH*, though both treatments have more protein expression at 4d treatment than the control group, HF group expressed less than that of the control group at 8d (Fig. 4B). For 4d treatment group, the p-value is 0.05, while that of the 8d treatment group is less than 0.0001. At 4d, LF expressed 1.41 fold and HF expresses 3.451 fold more than control; at 8d, LF expressed 3.93 fold and HF expressed 0.84 fold of control. For *Sox-2*, LF group has an initial expression that is lower than that of the control group, while HF cells have a higher expression (Fig. 4C). However, this switched at 8d treatment, with HF expressing less and LF expressing more than the control group. For 4d treatment group, the p-value is 0.1677, while that of the 8d treatment group is 0.0066. At 4d, LF expressed 0.875 fold and HF expresses 1.946 fold of the control expression; at 8d, LF expressed 3.366 fold and HF expresses 0.21 fold of control expression. For the three above proteins, when comparing 4d and 8d treatments with their respective mediums, LF has an upregulated expression for all three protein markers, while HF has a downregulated expression for all three. When tested for *vimentin*, nothing was

expressed for all three treatments. The opposite of vimentin is *E-cadherin*, where the level of expression remains the same for 4d and 8d treatment for all three mediums (Fig. 4D). For 4d treatment group, the p-value is 0.1780, while that of the 8d treatment group is 0.7397. LF cells expressed 1.673 fold of control at 4d and 1.342 fold at 8d. HF cells expressed 0.566 fold of control at 4d and 0.9283 fold at 8d. It should be noted that LF expressed more than that of the control group while HF expressed less. Lastly, for *Nanog*, HF cells treated for 4d did not express any *Nanog* proteins; however, at 8d, they started to express some, though still less than that of the control or LF cells (Fig. 4E). On the other hand, there is a decrease in *Nanog* expression for the LF cells from 4d to 8d; both expressed less than the control group (Fig. 4E). HF treated cells expressed little to no *Nanog* expression, while LF expressed 0.9 fold of control at 4d and 0.7 at 8d. For 4d treatment group, the p-value is 0.0055, while that of the 8d treatment group is 0.1143.

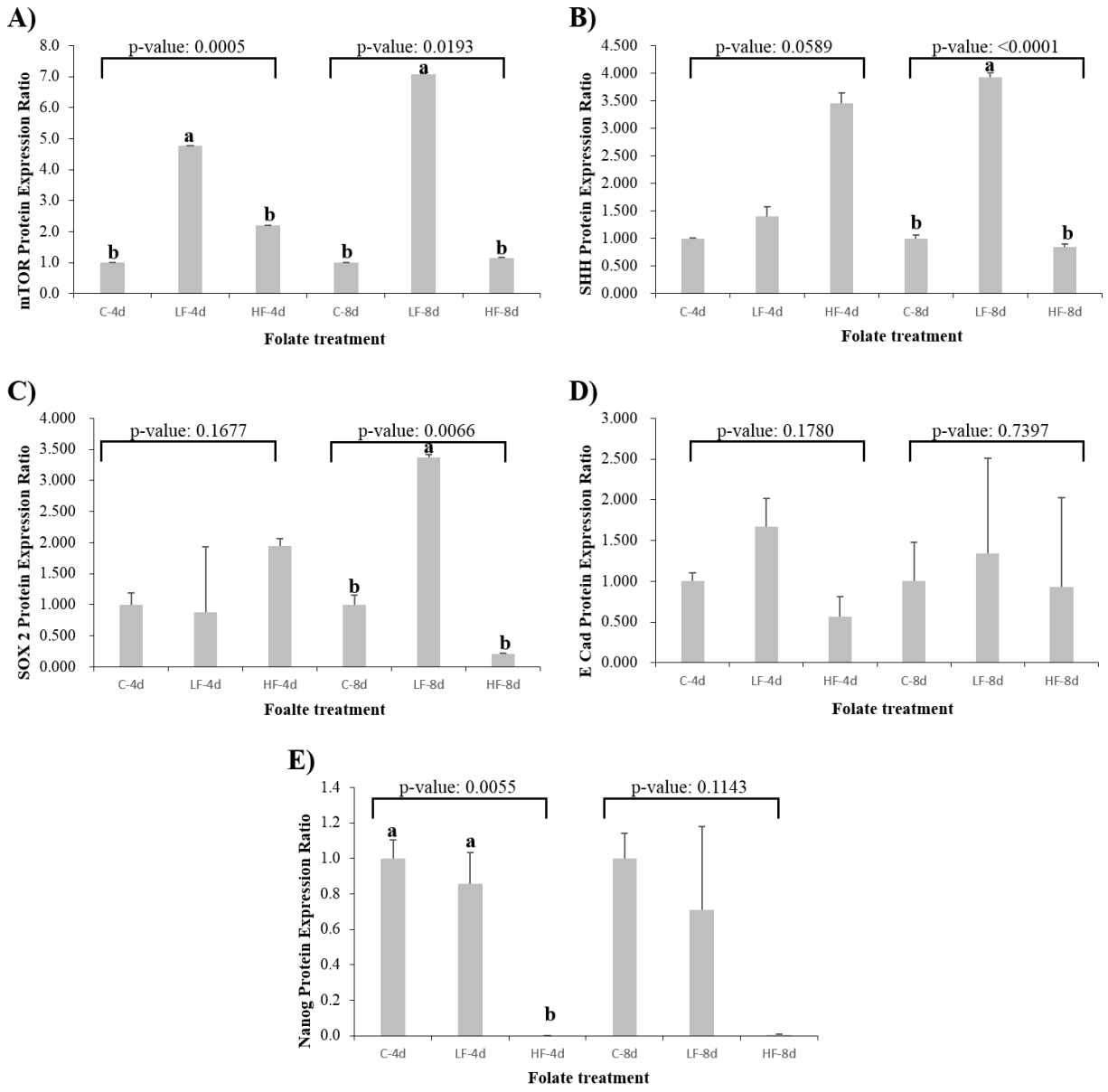


Figure 4. MCF-7 cells' expression of *mTOR*, *SHH*, *SOX-2*, *E-Cadherin*, and *Nanog*. MCF-7 cells were subjected to 4d and 8d treatments in LF, control, and HF mediums. The cells were collected to test for their protein expressions. The p-values are calculated using SAS9.4 software's One-way ANOVA analysis. Different superscripts are significantly different at P<0.05 by Duncan's multiple-range test. **(A)** *mTOR* target protein. **(B)** *SHH* target protein. **(C)** *Sox-2* target protein. **(D)** *E-cadherin* target protein. **(E)** *Nanog* target protein.

Discussion

LF, HF, and control mediums contain specific folic acid concentrations that reflect the average folate concentrations in cancer patients' blood serum who had either undergone chemotherapy, taken FA supplementation, or left untreated. Shahab-Ferdows *et al.* suggested that the average folate concentration of the serum among non-treated patients is around 2.2 μM [25], while Lamm *et al.* recommended to that patients prescribed with FA supplements have an average folate concentration of 50 μM of folic acid; those who had undergone chemotherapy without FA supplements have an average of 10nM folic acid[26].

For LF-treated cells, the data illustrates that the change in microenvironment to FD would cause the breast cancer cells to experience cell arrest, as shown through the lower doubling time compared to that of the control group (Fig. 1). After 2d depletion, the parameters given by Celígo indicate that LF-treated cells formed less and smaller tumorspheres than the control group, demonstrating that most MCF-7 cells had not transitioned into CSCs after only 2d depletion; the effects of the change in microenvironment had not kicked in (Fig.1). It is important to note, however, that some cells did transition into CSCs because some tumorspheres were formed during the oncospheroid cultivation. After 4d depletion, Fig. 2A depicts that more of LF cells had transitioned into CSCs and formed tumorspheres that are larger than the control group at a more rapid speed. After 8d depletion, even more tumorspheres were present just 3d cultivated in the oncospheroid medium; hence, 8d depletion caused more cells to transition into CSCs. The formation of tumorspheres confirms the self-renewal characteristics of CSCs. This trend is further illustrated through the expression of the stemness markers. The difference in the tumorspheres grown after 2d, 4d, and 8d depletion insinuates the idea that the timing of the depletion can cause a differential difference. As Murakami *et al.* identified, *mTOR* is essential for the growth and proliferation in stem cells; the inactivation of *mTOR* would impair its self- renewal capability [28]. LF-cells treated for 4d and 8d had more expression of *mTOR*, *SHH*, and *Sox-2* than that of the control group, suggesting that more MCF-7 cells transitioned into CSCs and expressed stemness markers. Interestingly, the pattern between 4d and 8d suggests that the longer the cells are exposed to LF condition, more cells would become CSCs and possibly metastasize. This correlates with Heiden *et al.'s* finding as they concluded that the *SHH* pathway maintains the CSC self-renewal characteristics through Gli1-induced *Snail* expression [29]. As suggested by Xia and Xu, the *mTOR* signaling pathway is the master regulator for cancer:

the activation of the *mTOR* complexes increases mRNA translation, protein synthesis, and cellular proliferation [15].

For HF-treated cells, the data characterizes the expedition of cell proliferation caused by the increase in FA present in the microenvironment as the doubling time of HF cells is significantly lower than that of the control group. After 2d supplementation, the parameters provided by Celígo signify that many of the MCF-7 cells have already transitioned into CSCs because the volume, area, and perimeter of the tumorspheres increased with the fastest pace. Fig. 3A shows the drop in the count of tumorspheres present between 4d and 7d of cultivation in the oncospheroid medium. This is because many tumorspheres come together and form bigger tumorspheres, as shown through the significant increase in spheres' sizes during the period (Fig. 3A). This similar trend is reinforced with cells 4d supplementation of HF (Fig. 3A). Though at 3d, HF tumorspheres have similar sizes and numbers with that of LF; starting from 6d, the number and size of the HF tumorspheres present a significant difference (Fig. 3a and 3C). The rapid growth of tumorspheres validates the idea that more of HF cells transitioned into CSCs as seen through their self-renewal characteristics. This pattern is once again described through the cells with 8d supplementation of HF (Fig. 3A and 3C). This correlates Manshadi *et al.* results, where she concluded that folic acid supplementation significantly promoted the progression of the sentinel mammary tumors and was associated with higher tumor weight, volume, and area when compared to the control diet [30]. Furthermore, HF-cells have an overexpression of *mTOR*, *SHH*, and *Sox-2* after 4d treatment; that expression decreased at 8d treatment, revealing that the expression of those stemness markers peak in 4d, where they will activate the signals in the downstream direction (Fig. 4).

For *SHH* and *SOX-2* expressions, only 8d treated cells presented significant difference, revealing that the longer cells are exposed to a change in microenvironment, whether it is LF or HF, the more impact the change has on the cancer cells. The difference in the expression of *E-cadherin* among the various treatments proves to be insignificant (Fig. 4D). One potential reasoning is because *E-cadherin* is not the principal epithelial marker for MCF-7. Zhang *et al* tested for MCF-7 cells' EMT with 4 types of markers: *E-cadherin, β-cadherin, N-cadherin, and vimentin.* Among the four, *β-cadherin, N-cadherin, and vimentin* present drastic difference in between treatments, while *E-cadherin* expression levels decreased slightly [31] Given the fact that the two treatments did not modulate *Nanog* overexpression of the MCF-7 cells (Fig. 4E), the data suggest that LFMS and FA

supplementation may epigenetically reprogram the stemness gene signatures, such as the *Nanog* marker, instead of being activated by the Hedgehog signaling [32].

The above findings reinforce the lines of experimental data that suggest that the timing and doses of folate does impact the transition of cancer cells into CSCs and promote tumorigenesis. Ulrich concluded that an increase in folate before the formation of tumor can prevent tumor development, but supplementation with synthetic folic acid after the lesions are present may enhance the progression, which correlates with this research's conclusion [33].

However, there exist a few limitations to the experiments. Analysis of tumor-folate interaction with CSC-associated markers by signature gene expression may not reflect the authentic relationships in the human breast cancer tissue. Due to the unavailability of tumor specimens further studies are needed to confirm the interrelationships. Furthermore, each patient's folate level may differ, depending on one's chemotherapy and folate supplementation; hence, the folate concentration of the mediums used in this research are rough estimations of the general population. Though *SHH* and *mTOR* signaling pathways appeared to be controlling the breast cancer cells, and thus the CSCs, in this research, *in vitro* environment might not mimic the complex human system. Prospective designed studies analyzing the pathways in human bodies are warranted.

Conclusion

The data shows that both high folate and low folate microenvironments promote *in vitro* tumor formation and transition into CSCs. This increases the chance of metastasis of breast cancer cells. Short-term exposure to folate depletion promotes transition of cancer cells into CSCs, though the percentage of cells undergoing transition is low. Long-term exposure, on the other hand, differentially induces the cancer cells to grow into CSCs, indicating that LF microenvironment expedites etastasis rate. Similarly, both a short-term and long-term treatment with FA supplementation cause MCF-7 cells to transition into CSCs, as shown through the expression of stemness markers and the significant increase in tumorsphere formation. These findings convey that new treatment for breast cancer patients is needed as both chemotherapy and FA supplementation to counteract the folate imbalance would undo the effect of therapeutic drugs.

Further studies are needed to investigate how the transition of cancer cells into CSCs from cancer cells is regulated and the specific mechanism related to the two microenvironments. Also, it is necessary to test the conclusion *in vivo* by injecting *in vitro* tumorspheres into animal bodies to see if metastasis occurs. With appropriate findings, novel therapeutic strategies for breast cancer patients can be personalized in accordance to their nutritional status to minimize cancer metastasis.

Work Cited

[1] Jin X and Mu P. "Targeting Breast Cancer Metastasis." *Libertas Academica* 9.1 (2015): 23-24.

[2] Sharma G, Dave R, Sandaya J, Sharma P, Sharma K. "Various Types and Management of Breast Cancer: An Overview." *Journal of Advanced Pharmaceutical Technology and Research* 1.2 (2010): 109-126.

[3] Liotta LA and Kohn EC. "The Microenvironment of the Tumor-host Interface." *Nature* 411.6825 (2001): 375-379.

[4] Lapidot T, Sirard C, Varmoor J, Murdoch B, Hoang T, Caceres-Cortes J *et al.* "A Cell Initiating Human Acute Myeloid Leukaemia after Transplantation into SCID Mice." *Nature* 367.6464 (1994): 645-648.

[5] Cukierman E and Bassi DE. "The Mesenchymal Tumor Microenvironment: a Drug-resistant Niche." *Cell Adhesion and Migration* 6.3 (2012): 285-296.

[6] Curtin J and Lorenzi M. "Drug Discovery Approaches to Target Wnt Signaling in Cancer Stem Cells." *Oncotarget* 1.7 (2010): 552-566.

[7] Maenhaut C, Dumont JE, Roger PP, and Van Staveren WCG. "Cancer Stem Cells: a Reality, a Myth, a Fuzzy Concept or a Misnomer? An Analysis." *Carcinogenesis* 31.2 (2010): 149-158.

[8] Cao L, Zhou Y, Zhai B, Liao J, Xu W, Zhang R *et al*. "Sphere-forming Cell Subpopulations with Cancer Stem Cell Properties in Human Hepatoma Cell Lines." *BioMed Central Gastroenterology*, 11, 71, 2011.

[9] Velasco-Velázquez MA, Popov VM, Lisanti MP, Pestell RG. "The Role of Breast Cancer Stem Cells in Metastasis and Therapeutic Implications." *The American Journal of Pathology*, 179, 1, 2011, 2-11.

[10] Tang DG. "Understanding Cancer Stem Cell Heterogeneity and Plasticity." *Nature Cell Research* 22 (2012): 457-472.

[11] Shen CN, Burke ZD, and Tosh D. "Transdifferentiation, Metaplasia and Tissue Regeneration." *Organogenesis* 1. 2 (2004): 36-44.

[12] Puisieux A, Barbletz T, and Caramel J. "Oncogenic Roles of EMT-inducing Transcription Factors." *Nature Cell Biology* 16 (2014): 488-494.

[13] Mukherjee S, Frolova N, Sadlonova A, Novak Z, Steg A, Page GP *et al.* "Hedgehog Signaling and Response to Cyclopamine Differ in Epithelial and Stromal Cells in Benign Breast and Breast Cancer." *Cancer Biology and Therapy* 5.6 (2006): 674-683.

[14] Hadjimichael C, Chanoumidou K, Papadopoulou N, Arampatzi P, Papamatheakis J, and Kretsovali A. “Common Stemness Regulators of Embryonic and Cancer Stem Cells.” *World Journal of Stem Cells* 7.9 (2015): 1150-1184.

[15] Xia P and Xu XY. “PI2K/Akt/mTOR Signaling Pathway in Cancer Stem Cells: from Basic Research to Clinical Application.” *American Journal of Cancer Research* 5.5 (2015): 1602-1609.

[16] Chiarugi A, Dolle C, Felici R, and Ziegler M. “The NAD Metabolome – a Key Determinant of Cancer Cell Biology.” *Nature Reviews Cancer* 12.11 (2012): 741-752.

[17] Sun M, Yang C, Zheng J, Wang M, Chen M, Le DQS *et al.* “Enhanced Efficacy of Chemotherapy for Breast Cancer Stem Cells by Simultaneous Suppression of Multidrug Resistance and Antiapoptotic Cellular Defense.” *Acta Biomaterialia* 28 (2015): 171-182.

[18] Eyler CE and Rich JN. “Survival of the Fittest: Cancer Stem Cells in Therapeutic Resistance and Angiogenesis.” *Journal of Clinical Oncology* 26.17 (2008): 2839-2845.

[19] *Stover PJ*. “One-Carbon Metabolism-Genome Interactions in Folate-Associated Pathologies.” *The Journal of Nutrition* 139.12 (2009): 2402-2405.

[20] Zhao R, Matherly LH, and Goldman ID. “Membrane Transporters and Folate Homeostasis; Intestinal Absorption Transport into Systemic Compartments and Tissues.” *Expert Reviews in Molecular Medicine Journal* 11.4 (2009).

[21] Visentin M, Zhao R, and Goldman ID. “The Antifolates.” *Hematology/Oncology Clinics of North America* 26.3 (2013): 629-632.

[22] Stockstad, E.L.R. “Historical Perspectives on Key Advances in the Biochemistry and Physiology of Folates.” *Current Issues in Clinical Nutrition* 13 (1990).

[23] Wien TN, Pike E, Wisloff T, Staff A, Sigbjorn S, and Klemp M. “Cancer Risk with Folic Acid Supplements: a Systematic Review and Meta-analysis.” *BMJ Open* 2.1 (2012).

[24] Yi K. “Folate and Colorectal Cancer: an Evidence-based Critical Review.” *Molecular Nutrition and Food Research* 51.3 (2007): 267-292.

[25] Shahab-Ferdows S, Engle-Stone R, Hampel D, Ndjebayi A, Nankap M, Brown K *et al.* “Risk Factors for Folate Deficiency Differ from Those for Vitamin B12 Deficiency in Cameroonian Women and Children.” *The FASEB Journal* 28.1 (2014).

[26] Lamm N, Maoz K, Bester AC, Im MM, Shewach DS, Karni R *et al.* “Folate Levels Modulate Oncogene-induced Replication Stress and Tumorigenicity.” *EMBO Molecular Medicine* 7.9 (2015): 1138-1152.

[27] Strickland KC, Krupenko NI, and Krupenko SA. “Molecular Mechanisms Underlying the Potentially Adverse Effects of Folate.” *Clinical Chemistry and Laboratory Medicine* 51.3 (2013).

[28] Murakami M, Ichisaka T, Maeda M, Oshiro N, Hara K, Kiyama H et al. “mTOR Is Essential for Growth and Proliferation in Early Mouse Embryos and Embryonic Stem Cells” *Molecular and Cellular Biology* 25.12 (2004): 6710-6718.

[29] Heiden KB, Williamson AJ, Doscas ME, Ye J, Wang Y, Liu D *et al.* “The Sonic Hedgehog Signaling Pathway Maintains the Cancer Stem Cell Self-Renewal of Anaplastic Thyroid Cancer by Inducing Snail Expression.” *The Journal of Clinical Endocrinology and Metabolism* 99.11 (2014).

[30] Manshadi SD, Ishiguro L, Sohn KJ, Medline A, Renlund R, Croxford R *et al.* “Folic Acid Supplementation Promotes Mammary Tumor Progression in a Rat Model.” *PLoS One* 9.1 (2014).

[31] Zhang X, Li Y, Zhang Y, Song J, Wang Q, Zheng L *et al.* “Beta-elemene blocks epithelial-mesenchymal transition in human breast cancer cell line MCF-7 through Smad3-mediated down-relation of nuclear transcription factors.” *PLoS One* 8.3 (2013).

[32] Chatel G, Ganeff C, Boussif N, Delacroix L, Briquet A, Nolens G *et al.* “Hedgehog Signaling Pathway is Inactive in Colorectal Cancer Cells Lines.” *International Journal of Cancer* 121.12 (2007): 2622-2627.

[33] Ulrich CM. “Folate and Cancer Prevention: a Closer Look at a Complex Picture.” *The American Journal of Clinical Nutrition* 86.2 (2007): 271-273.

【評語】090010

This research tested the effect of folic acid in breast cancer and breast cancer stem cells. Author measured the properties of cell proliferation, tumor sphere, and stem cell markers. She has done a lot of work and the results are amazing. She found that cancer cells eventually grow despite low or high dose of folate treatment. She has also done a lot of Western blot analysis testing the expression of stemness genes, mTOR, Shh, and SOX2. This is very impressive for a high school student.

The only down side is that the results are less striking so it will lead to a warning to people who have high hopes about folate in cancer prevention or treatment. Data presentation methods are also not ideal, but these problems are not fatal.