

The Plasticity of Gastric Cancer Cell Lines

Muhammad Aizat Md Hawari

Division of Surgery and Cancer

Faculty of Medicine

Imperial College London

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Declaration of Originality

I declare that all research presented in this thesis is represents my own original work, unless otherwise stated. All other work has been appropriately referenced. I confirm that this thesis has been composed by myself and not been previously submitted to any degree or any other institution.

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وَإِذَا مَرَضْتُ فَهُوَ يَشْفِينِ ﴿٨٠﴾

— *And when I am ill, it is He who cures me*
(*Al Quran*, 26:80)

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Abstract

Cell heterogeneity is a known feature of cancer cell populations. Because of this, cell population behaviour in response to a particular environment may not be replicated in a specific subpopulation. This has prompted a new hypothesis: that cancer cells can undergo phenotypic change to acquire a stem-like cell phenotype. Thus, to determine which sub-population that displays a specific biological function and whether the environment could influence the plasticity of a group of cells within the population is essential. To investigate the potential influence on cell's plasticity, human gastric cancer cell line SNU-1 was treated with benzo[a]pyrene (BaP), a potent IARC Group I human carcinogen, to induce a phenotypic response. BaP-AhR associated genes (CYPs) (*i.e.* *CYP1a1*, *CYP1b1*, *AHR*, *AHRR*, *ARNT*) and critical genes associated with epithelial to mesenchymal transition (*i.e.* *Snail*, *TWIST1*) and stemness markers (*i.e.* *SOX2*, *OCT3/4* and *NANOG*) were examined. We suspected that subpopulations within SNU-1 were involved. Therefore, flow cytometry was used to observe the expression of CD24 and CD166. In unperturbed culture, enriched CD24⁺ and CD166⁺ respective cells were found to revert to their original expression phenotype after 72 hours. However, with BaP-treatment, the sorted cells increased surface expression of CD24 and CD166, respectively. Each sorted population (CD24⁺, CD166⁺ and CD24⁺CD166⁺) showed different regulation of genes associated with stemness and BaP-induced phenotypic change. *CTNNB1*, a target gene of Wnt/ β -catenin signalling pathway, *MYC*, and EMT-inducer genes were upregulated in the unsorted SNU-1 population and sorted CD24⁺ (BaP 10⁻⁷M to 10⁻⁵M) while remained unchanged in CD166⁺ and CD24⁺CD166⁺ populations. Moreover, the induced expression of AhR-related and stemness genes in the sorted CD24⁺ population, potentially indicates that this subpopulation probably contributes to the plasticity of SNU-1. BaP-treatment also mediates changes in stemness expression and enhanced its migrative and invasive capabilities.

Thus, the co-existence and interaction of subpopulations within SNU-1 could play a role in cell plasticity and in sustaining key signalling pathway responses and stemness when exposed to BaP. Our data suggest that cell heterogeneity and plasticity of subpopulations within gastric cancer cells could contribute to BaP-induced gastric carcinogenicity

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List of abbreviations

Abbreviation	Meaning
2D	Two-Dimensional
ABCG2	ATP-binding cassette super-family G member 2
AhR	Aryl Hydrocarbon Receptor
APC	Allophycocyanin
ARNT	Aryl Hydrocarbon Nuclear Translocator
AhRR	Aryl Hydrocarbon Receptor Repressor
ANOVA	One-Way Analysis of Variance
BaP	Benzo[a]pyrene
BDPE	B[a]p-7,8-dihydrodiol-9,10-epoxide
CD	Cluster of Differentiation
CSC	Cancer Stem Cell
CT	Cycle Threshold
CTNNB1	Gene encodes for Catenin beta-1 protein, also known as β -catenin
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DT	Diffuse-Type
EMT	Epithelial-to-Mesenchymal Transition
FCR	Fc receptor is a protein found on the surface of certain cells
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence Minus One
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gastric Cancer
GCSC	Gastric Cancer Stem Cells
IT	Intestinal-Type

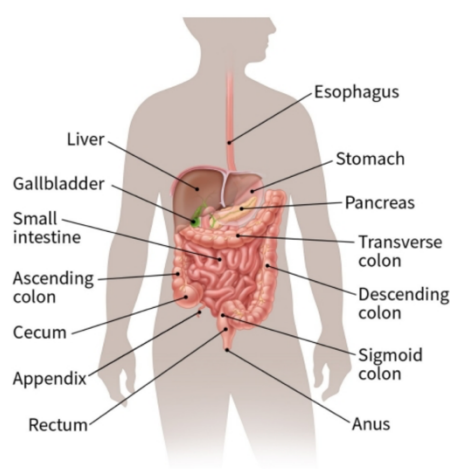
LS Column	Proprietary column by Miltenyi Biotec, designed for positive selection of cells or depletion of strongly magnetically labelled cells.
MET	Mesenchymal-to-Epithelial Transition
MN	Micronucleus/Micronuclei
mRNA	Messenger RNA
NTC	Non-Tumorigenic Cells
PE	Phycoerythrin
PET	Polyethylene Terephthalate, used to make the microporous membrane inserts
PBS	Phosphate Buffered Solution
PBA	Protein Blocking Agent
QPCR	Quantitative-Polymerase Chain Reaction
QRT-PCR	Quantitative Reverse Transcription-Polymerase Chain Reaction
RPMI 1640	Growth medium used in cell culture
RNA	Ribonucleic Acid
SD	Standard Deviation
SNAIL	Zinc finger protein SNAIL or Snail
TC	Tumorigenic Cells

1 Introduction and Literature Review

1.1 Carcinogenesis

Carcinogenesis begins in healthy somatic cells which undergo mutations and acquire significantly altered proliferation. Under the influence of DNA damage or genetic instability causing the cells to undergo mutation, the mutated cells can begin displaying enhanced proliferation, or acquire resistance to cell death, then the emerging cell clones could manifest as small hyperproliferating populations, or even adenoma or adenocarcinoma. The hallmarks of cancer have been thoroughly discussed by Hanahan and Weinberg [1] who stated that cancer mainly encompasses hallmarks including sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death.

1.2 Common types of cancer



According to the American Cancer Society, breast cancer is the most common type of cancer in the United States, followed by lung and gastrointestinal types of cancer¹. Similarly, in the United Kingdom, breast cancer is the most common cancer followed by gastrointestinal cancer. Although there are about 200 types of cancer, four types – breast, prostate, lung and bowel – accounted for 53% of all new cases in the UK (2015)². Gastrointestinal (GI) cancer refers to a group of cancers that affects the

Figure 1.1: Human digestive system

¹ American Cancer Society: Cancer Facts and Figures 2018. Atlanta, Ga: American Cancer Society, 2018. Accessed: November 2018.

² Cancer Research UK: Cancer incidence for common cancers. Online accessed: November 2018.

digestive system (see figure 1.1)³. This system consists of oesophagus, gallbladder, biliary tract, liver, pancreas, stomach, small intestine, bowel (large intestine, colon and rectum), and anus.

1.3 The gastric epithelium

Stomach cancer, or gastric cancer, begins in the stomach. When food enters the body, the oesophagus delivers food to the stomach. The oesophagus connects to the stomach at the gastroesophageal (GE) junction. The secretion of gastric juice assists the digestion process in the stomach. Histologically, the human gastric zone is divided into three zones: Cardia, Corpus, Fundus, Antrum & Pylorus [2] (see figure 1.2). The gastric mucosa is also surrounded by subepithelial myofibroblasts [3]. In each zone, the gastric unit is composed of a long tubular gland that partitions into the pit, isthmus, neck, and base regions.

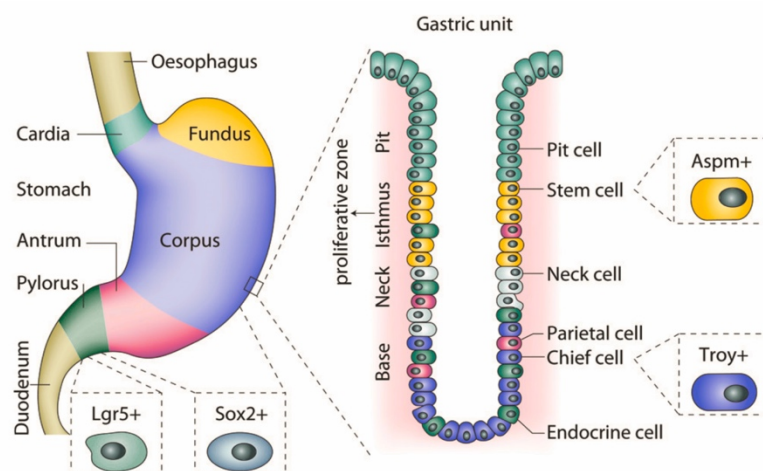


Figure 1.2: Schematic diagram showing the macroscopic anatomical regions and microscopic cellular constituents of the glandular stomach. Adapted from Watson *et al.* [4].

1.3.1 Gastric cancer

Stomach cancer or gastric cancer (GC) ranks as the 6th leading cause of death in cancer patients [5]. Although deadly, in the UK, 10 or more years' survival is around 15%; however, with preventive measures, the occurrence of GC can be reduced by 75% [5].

³American Cancer Society: Stomach Cancer. "About and Statistics" Online accessed: November 2018.

In GC, *Helicobacter pylori* (*H. pylori*) infection, diet and lifestyle have been attributed to the development of cancer [6]. Studies of GC have long used the histological classification by Lauren's criteria that histologically characterised GC into two major histologic subtypes of well-differentiated (intestinal type, (IT)), poorly differentiated components (diffused type (DT)) cells [7]-[11]. This classical histologic classification, or Lauren P. classification was coined in 1965 when the author observed that the IT was structurally similar to colonic cancer but the other type, the DT, was seen only in the stomach [9]. Modern studies have found that persistent *H. pylori* infection have been strongly associated with IT GC [12]. The infection causes genetic and molecular changes that lead to activation of critical oncogenes, along with inactivation of tumour suppressors, which causes DNA repair failure and overexpression of growth factors thus leading to severe atrophic gastritis and intestinal metaplasia [13]. On the other hand, the DT GC is generally related to cellular decohesion, poor differentiation, resistance to treatment, and poorer patient outcome [14].

Regardless of different causes, IT and DT have distinct carcinogenetic pathway or phenotypically different. By correlating their histologic characteristics with molecular profiling and clinical behaviour, scientists and clinicians seek to understand the clinicopathologic entity of each subtype to devise appropriate interventions, or improved treatments [15].

1.3.1.1 Cancer cell lines

Cancer cell lines are cultures developed beyond primary cell culture (cells from parental animal, or human tissues). Ideally, cell line models should mirror the properties of primary cancers (from the original organ, or tissue where it began) in terms of maintenance of histopathology when transplanted into immune-deficient mice, genotypic and phenotypic characteristics, gene expression, and drug sensitivity [16]. However, since fast-growing cell lines optimal for an *in vitro* growth are selected from poorly differentiated tumours, the cell lines may inevitably not always replicate the reality found in the clinical disease [16]. Thus, although the chosen cell lines should replicate the genotypic and phenotypic characteristics of primary cancers as closely as possible [17], it is essential to note that not all cancer cell lines will reflect the tumour from which it was derived. For instance, Nelson-Rees and colleagues showed that changes in cell line's characteristics could also occur in cell cultures with high passage number [18].

Generally, cancer cell lines may exist either as adherent cultures, or they may grow in "suspension." Most cell types will adhere to a "substrate" such as plastic or glass, and proliferate as a monolayer, while suspension cultures do not attach to a substrate and therefore grow floating in medium [19]. It was reported that with the increasing number of passages, cell lines lose specific differentiation characteristics as well as changes in phenotype [16], [20], [21].

Nonetheless, many gastric cancer studies have been conducted on immortalized GC cell lines [22]-[24]. Typically, these cells are a population taken from a patient and grown *in vitro* for prolonged periods. The ability to proliferate indefinitely is caused by genetic change⁴, or mutation in which the cells successfully evade cellular senescence and can maintain cell division, providing a consistent renewable source of cell material for study. This unique ability can be acquired naturally or via induction of specific experimental objectives [16], [29]. Thus, it is essential to consider limitations of studies of derived cell lines in terms of genomic and transcriptomic changes in comparison to studies of primary tumours. Gazdar and colleagues in a review of lung cancer cell lines pointed out that, in general, genetic and epigenetic changes, including driver mutations, are mostly preserved [26]. Nevertheless, through exploring the whole transcriptome expression profile, several studies in NC160 (the panel of 60 human cancer cell lines; [27], [28], and gastric cell lines [29], found that there are strong positive correlations and minor genetic differences between cell lines and primary tumours.

1.3.1.2 The growth of cells in subculture

The cells pass through several distinct stages of growth at every subculture. It begins with a lag phase, where the culture grows relatively slowly (refer Figure 1.3). Additionally, the exchange of growth factors will accelerate the growth rate and induce a high percentage of cell division [17]. The initial cells seeding density plays a critical role too: the higher the cell density, the more rapidly the culture will grow [17]. The cells will exhibit log phase kinetics during the most rapid phase of exponential growth. Finally, when the cells reach maximum confluency, when there is relatively high use of media components, the growth rate will reach saturation density, which will be referred to as plateau phase. Beyond that, the cells will dwindle in number and decline (refer Table 1.1).

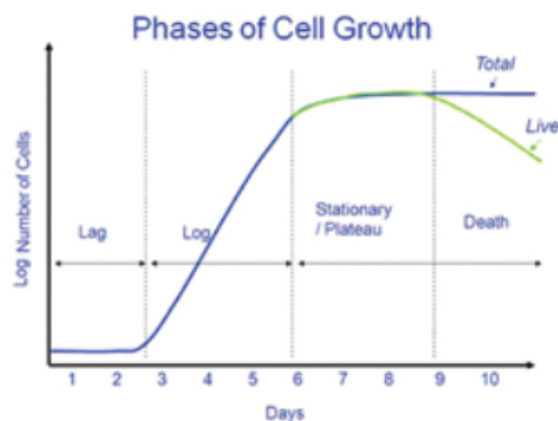


Figure 1.3 Cell culture growth curve example.

(Adapted from <https://www.sigmaaldrich.com/technical-documents/protocols/biology/cell-types-culture.html>)

Lag Phase	Cells do not divide at this stage but adapt to culture conditions. The length of this phase will depend upon the cells' growth phase and seeding density.
Logarithmic (Log) Growth Phase	An exponential increase in cell density due to active cells proliferation. Cell line will be the most viable; therefore, cellular function is best accessed at this phase. Different cell lines will also show varied cell proliferation kinetics. Thus, the population doubling time is best determined at this phase. Delaying passaging cells at this phase will lead to over-confluency, apoptosis and senescence.
Plateau (or Stationary) Phase	Due to confluency, cell proliferation slows down. Cells are most susceptible to injury at this stage.
Decline Phase	Viable cells are reduced, cells undergo cell death, not necessarily due to lack of nutrition but in a natural progression of the cellular cycle.

Table 1.1: A typical growth curve for cultured cells displays a sigmoid pattern of proliferation
(Adapted from <https://www.sigmaldrich.com/technical-documents/protocols/biology/cell-types-culture.html>)

1.3.1.3 Gastric cancer cell lines: SNU-1, KATO-III, NCI-N87

Since 1982, SNU (Seoul National University) cell lines have been obtained from Korean cancer patients. One hundred and nine of 180 tumour cell lines taken from 2,200 cancer tissues have been characterized and described in detail [22], [30], [31]. The cell lines used in this thesis, namely SNU-1 and NCI-N87, were isolated from primary gastric carcinoma of the stomach epithelial and hepatic metastatic site respectively [22]. SNU-1 is a suspension type of cell line with multi-cell cellularly discohesive aggregates that are morphologically epithelial [31], [32], whereas NCI-N87 is an adherent type of cell line consisting of tightly knit epithelial cells [32]. Both cell lines have been used in various types of experiments such as anti-cancer drug

screening [33], the study on multidrug resistance [34], the effect of methylation [35], KRAS mutation [36] and invasion assays of GC cells [37], [38]. The KATO-III cell line was established in Japan and derived from the pleural effusion (from the metastatic site at supraclavicular and axillary lymph nodes and Douglas cul-de-sac) [25], [39]. KATO-III signet ring cell carcinoma line was reported to have a genomic deletion of *P53*, amplification of *K-SAM* and *C-MET* oncogenes and mutation of *E-cadherin* [40]. Table 1.2 summarises the characteristics of these cell lines.

*Table 1.2 Origin, culture morphology and growth characteristic of gastric carcinoma cell lines used in this study. *OTD: Original tumour differentiation; T: Tumorigenicity; n/a: information not available. References: [22], [40]-[42])*

Cell lines	Origin				Tumour Site	OTD	Substance Adherence	T	Putative Markers	GC Classification
	Age	Sex	Country	Prior Therapy						
SNU-1	44	M	Korea	None	Stomach	Poor	Suspension	+	<i>SOX2</i>	DT [43]
KATO III	55	M	n/a	n/a	Pleural effusion	Well	Suspension & adherent	+	<i>CD133</i> , <i>CD44</i> , <i>SOX2</i> ,	DT [44]
NCI-N87	n/a	M	USA	None	Liver	Well	Adherent	+	<i>CD44</i> , <i>SOX2</i>	IT [44]

1.4 Stem cells

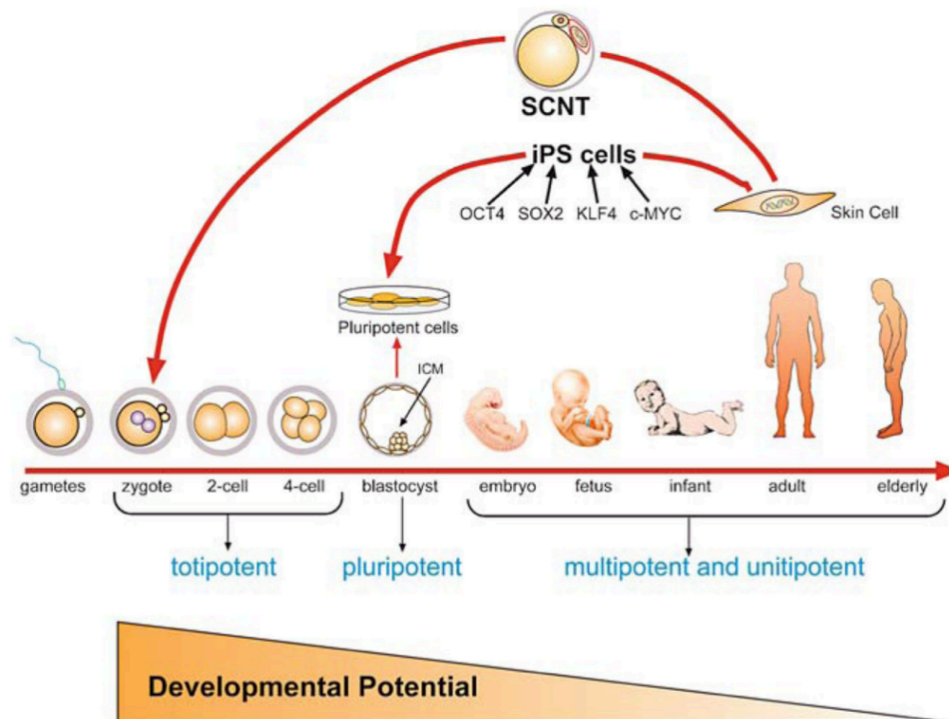


Figure 1.4: Development and reprogramming. From a single cell, the zygote and each blastomere of the early embryo act as totipotent cells in the series of ontogenesis processes. Developmental potential of each cell type plays a crucial role as a result of potential gradually declining from the earliest developmental stage to maturity: totipotent cells become pluripotent, multipotent and unipotent to finally differentiate to somatic cells. Interestingly, genetic manipulation approaches such as somatic cell nuclear transfer (SCNT) and direct reprogramming can potentially restore the developmental potential of somatic cells.⁴

By definition, stem cells (SC) are cells that can propagate themselves by self-renewal mechanisms while maintaining its undifferentiated state, and can differentiate into mature, or specialised cells of specific tissues [45], [46]. While the population of stem cells is low in each tissue, it can be carefully purified and isolated. For instance, mesenchymal stem cells can be isolated from placental tissues [47]; hematopoietic stem cells (HSCs) – from mice and humans [48], [49], human epidural cells [50], and mouse bone marrow [51]. The key to stem cell function is their self-renewal ability, which allows the tissues of an organ to sustain themselves throughout the lifetime of the organism [52].

While the differentiation potential for stem cells to become specialized cells varies (Figure 1.4) all stem cells types must regulate and maintain the relative balance between self-renewal and differentiation [46]. Totipotent cells can construct a complete and viable organism [53]. In the

⁴ Adapted from: Mitalipov, S., & Wolf, D. (2009). Totipotency, pluripotency and nuclear reprogramming. *Advances in Biochemical Engineering/Biotechnology*, 114(1), 185–199.

early days of fertilization, these cells can form the embryonic cells. Descended from totipotent cells, the embryonic stem cells become more specialised to form any of the germ layers (endoderm, mesoderm, ectoderm), and are considered as pluripotent [54]. These pluripotent cells have lineage restricted potential that can only generate tissue-specific multipotent cells from the germ layer they came from (Figure 1.4) [46]. Adult stem cells (or somatic stem cells) are undifferentiated cells that have self-renewing and multipotency properties, and they are responsible for replenishing or regenerating cells from their tissue of origin [55].

1.4.1 Gastric stem cells

Based on the histology of macroscopic anatomical regions and cellular constituents of the glandular stomach (Figure 1.2), the gastric body consists of three major regions: the cardia, the corpus, and the antrum/pylorus. The various gastric units from the inner gastric mucosa contain various cell types residing in the pit, isthmus, neck, and base. A recent mouse study found that 86% of proliferating cells occurred in the isthmus. With lineage-tracing, the study also found that in order to maintain the gastric unit, mature cells were continuously supplied by stem cells located in the isthmus and antrum region [56]. *TROY*⁺ chief cells in the corpus, *LGR5*⁺ or *SOX2*⁺ cells within the pyloric glands and *ASPM* in the proliferative isthmus zone have been previously proposed as molecular markers for gastric stem cells [57]-[59]. Previous studies on the gastric unit show the isthmus as the gastric stem cell zone: different gastric stem cells in the isthmus likely supply a continuous bidirectional regeneration of differentiated gastric epithelial cells [60], [61]. Moreover, the gastric epithelium is continuously self-renewing, therefore as differentiated cells die, the new ones are replenished.

Gastric units are monoclonal (forming a clone from a single cell) in origin, therefore they can pass down mutation to all their differentiated progeny, which can lead to several issues such as metaplasia in the normal mucosa and gastric mucosa [62]. Thus, gastric epithelial cells can originate from a multi-potent stem or progenitor cells [60]. This is supported by the existence of multipotent stem cells in the rodent stomach, confirmed by lineage-tracing studies [63], [64], in which the investigators found that pluripotency of early embryonic cells is preserved and regulated by the transcription factor *SOX2* (an important pluripotency marker), which contributes to the formation of several epithelia, including gastric epithelia, during foetal development [64]. The residence and identity of gastric stem cells have attracted considerable interest because of their potential roles in physiological homeostasis and pathological

conditions, especially cancer. The most common stemness factors: *SOX2*, *OCT3/4*, and *NANOG* are regarded as crucial pluripotency regulators in progenitor cells⁵ and may contribute to tumorigenicity [42], [64], [66]-[70]. Table 1.3 summarizes the association of the respective stemness factor with pluripotency and tumorigenicity.

1.4.2 Stemness regulators: *SOX2*, *OCT3/4*, and *NANOG*

Although genetic control of the self-renewal ability of pluripotent, foetal, and adult stem cells is not well understood [71], recent findings have suggested the possible involvement of polycomb group protein BMI-1 [72], [73], the HMG box transcription factor *SOX9* [74], [75], telomere subunit *TERT* [76], and G-protein coupled receptor *LGR5* [58], [77] at the centre of it. These molecular regulators are observed in different adult stem cells; however, they are mostly absent in pluripotent stem cells. It was proposed that pluripotency genes such as *SOX2*, *OCT3/4* and *NANOG* [78]-[82] are expressed in adult stem cells; however, animal genetic studies showed that *OCT3/4* is not required to maintain adult stem cell self-renewal [83]. Another study indicated that *NANOG*, but not *OCT-4* and *SOX-2*, is expressed in cultured human adult mesenchymal stem cells (MSC) [84]. Despite the discrepancy in these findings, immunohistochemistry of 290 patients showed that strong correlation with *SOX2*-positive or *OCT3/4*-negative expression might be associated with lymph node metastasis, or lymphatic invasion and worse prognosis [68]. These transcriptional factors are also involved in hepatic cancer [85], tumorigenic potential in gastric cancer [86], and contribute to glioblastoma cancer recurrence [87] and colon cancer [67]. Therefore, the role of these stemness genes in human malignancy must be taken into serious consideration [64], [68], [88], [89].

Table 1.3: Stemness factor association with pluripotency in normal cells vs cancerous cells. Adapted from: [95],[74]

Stemness Factor	Association with pluripotency	Association with tumorigenicity
<i>SOX2</i>	Transcription factor expressed in undifferentiated pluripotent embryonic stem cells and germ cells during development. Together with <i>OCT-4</i> and	Promote cell proliferation and tumorigenesis of breast cancer.

⁵ Progenitor cells are already more specific than adult stem cells, in a way that they are limited to differentiation into a specific ‘target’ cells (the mid-point between adult stem cells and fully differentiated cells) [65].

	<i>NANOG</i> , it is necessary for the maintenance of pluripotent potential.	
<i>OCT3/4</i>	Transcription factor expressed in undifferentiated pluripotent embryonic stem cells and germ cells during healthy development. Together with <i>SOX2</i> and <i>NANOG</i> , it is necessary for the maintenance of pluripotent potential.	Implicated in self-renewal and tumorigenesis via activation of its downstream genes in cancer-stem like cells.
<i>NANOG</i>	Homeodomain-containing transcription factor essential for the maintenance of pluripotency and self-renewal in embryonic stem cells. The expression is controlled by a network of factors, including <i>SOX2</i> and the key pluripotency regulator <i>OCT-4</i> .	Associated with the early stage of carcinogenesis in pancreas cancer.

1.5 Cancer stem cells

1.5.1 What is cancer stem cell?

Cancer stem cell (CSC) is defined as a cell within a tumour that possesses the capacity to self-renew and that produces heterogeneous lineages of cancer cells that comprise the tumour. Identification of the CSC was first demonstrated by Dick *et al.*, who showed that tumorigenic properties could be attributed only to a minority population of leukaemia cells engrafted in immunodeficient mice [90]. The same concept and experimental approaches were tested for the first time in solid breast cancer tumour by Al-Hajj *et al.* [91]. They showed that breast tumour consists of a heterogeneous population of breast cancer cells based on surface marker expression. CSCs can be identified by expression of certain surface markers, which distinguish them from non-tumorigenic cells (non-CSC) [92], for example, gastric CSC can be identified using the cell surface marker CD44 [41], CD24 [93], CD133 [94] and CD166 [95]. Hence, CSCs represent a distinct cell population that can be identified and prospectively isolated from a wide variety of tumour tissues using CSC-specific markers and may express specific stemness genes [96].

The idea that “tumorigenic properties could be attributed only to a minority population” was tested by another study, when the investigators found that as few as 100 tumorigenic cells with CD44⁺CD24⁻ phenotype was able to form tumours in NOD/SCID mice, whereas tens of thousands of cells with alternative phenotypes failed [91]. Upon serial passage in culture, these tumorigenic population could form new tumours with phenotypically diverse non-tumorigenic cells [91]. Similar studies were also done in brain cancers [120] and colon cancers [110] – these studies reported that the sub-population identified with specific surface markers, sorted from primary tumours, were able to form tumours and recapitulate some of the heterogeneity of the primary tumours when transplanted in immunodeficient mice. Other studies have also indicated that individual cancer cells can regain the heterogeneity of the original tumour [97], [98]. Roesch *et al.* [99] suggested that a temporarily distinct subpopulation of cancer cells may mediate tumour maintenance in a dynamic process. Furthermore, CSCs are also known to have high level of plasticity in acquiring drug resistance phenotype. In a study, Settleman and his colleagues observed the dynamic regulation of phenotypic heterogeneity in drug tolerance, where drug-tolerant phenotype was acquired by cells within a sub-population that exhibit >100-fold reduced drug sensitivity and maintain cells viability *via* altered chromatin state [122]. There are evidences supporting the role of CSCs in drug resistance and cancer metastasis, thus

causative to the unfortunate consequences experienced by patients with pancreatic, brain, breast, colon, ovarian and prostate tumours [100]. CSCs differ from healthy stem cells in their increased resistance to anticancer drugs, tumour-initiating activity and metastatic ability, which can be driven by various factors such as chromatin alteration [124] miRNA machinery [101], genetic or epigenetic alteration to the cell's ABC transporters (such as ABCG2) that alter the cells sensitivity to drug. Clinically, the presence of ABC transporter contribute to cells resistance to multidrug treatment in chemotherapy [249].

ACBG2 is a member of ATP-binding cassette (ABC) transporter superfamily, and it is known to contribute to cell resistance to multidrug treatment in chemotherapy [249]. It is also understood to regulate self-renewal and is a recognised stem cell marker [82], [250]. Other researchers have reported that its expression is correlated with the degree of differentiation, and that *ACBG2* is highly expressed in poorly differentiated cells [94].

1.5.2 Immuno-phenotyping of cells or subpopulations

The cluster of differentiation or CD is a nomenclature used to identify and study cell surface molecules that acts as targets for immunophenotyping of cells or a population of cells [102], [103]. CD markers are widely used in stem cells research, such as to distinguish mesenchymal stem cells from fibroblast cells [47], [104]. In studies involving cancer stem cells (CSCs), CD markers are used to investigate the stemness population in primary gastric cancer tumour [105], survival analysis on hepatoblastoma patients [106], identify and characterise tumour-initiating cells in non-small-cell lung cancer [107], tumour invasion [108], and identify intestinal cancer stem cells [109] and many other studies [94], [110]-[112].

With the emergence of CSCs in the field of cancer research, many investigators are delving into studying this so-called "small population" within the heterogeneous population of cancer cells which may have aberrant self-renewal abilities. Therefore, to study this phenomenon in-depth, isolating this sub-population will be very crucial. There are a few ways CSC can be detected and isolated; the most common choice is to employ the use of magnetic flow cytometric cell sorting utilizing the specific surface markers to the type of tumour. It can be used for both positive and negative cell selection [41], [107]. Magnetic-activated cell sorting (MACS) uses magnetic and biodegradable microbeads covalently bonded to a specific

monoclonal antibody that permits the enrichment of cells that express the desired antigen. Its advantages include fast direct labelling and yields high purity cell populations.

Fluorescent-activated cell sorting (FACS) is an alternative isolation sorting method via utilization of fluorochromes with different emission wavelengths using direct or indirect immune fluorescence staining conjugated to primary or secondary antibodies. Although to isolate these populations, MACS appear to be simpler and require less technical complexity than FACS; it is mono-parametric. Thus, MACS cannot isolate cells via multiple markers simultaneously.

According to Tirino et al. (2012) [113], CSCs can be identified and isolated, employing four main methods:

- 1) Isolation of CSCs by flow cytometry according to CSC-specific cell surface markers.
- 2) Detection of side population phenotype by Hoechst 33342 (dye) exclusion.
- 3) Ability to grow as floating spheres in serum-free medium.
- 4) Aldehyde dehydrogenase (ALDH) activity.

None of the methods above can act as exclusive or a 'gold standard' to highlight specific CSC population. However, the cancer stem cells will be the cells that are: positive for 'stemness' markers, part of a side population, capable of forming spheres, and lastly, able to form tumours when injected into nude mice [114]. Based on table 1.4, it is possible that flow cytometry would be the best method to verify the phenotype of the cell lines being used.

Table 1.4 Methods for cancer stem cell verification and characterization

Method	Type of Verification	Advantages	Disadvantages
Flow cytometry	Phenotypic	- Reveals the phenotype of individual cells - Examines several markers simultaneously	- Requires expensive equipment - Requires skilled operator

		• Can be used to isolate specific populations (when equipped with a cell sorter) • High sensitivity • High-speed analysis • Can use gating to remove dead cells from analysis	• Potential cross-reactivity when using several antibodies • Potential autofluorescence • Cell sorting by FACS can be time-consuming and expensive
Immunofluorescence/ Immunocytochemistry	Phenotypic	• Reveals localization of marker proteins • Can assess multiple markers simultaneously • More efficient than Western blot analysis • Can use live or fixed cells	• Requires specialized equipment • Potential cross-reactivity when using multiple antibodies • Potential autofluorescence • Photo-bleaching • More time consuming than flow cytometry
Western Blot to detect stem cells and Differentiation Markers	Phenotypic	• High sensitivity • High specificity • Does not require specialized equipment	• Does not reveal the heterogeneity of the cell population • Low throughput
Protein Array	Phenotypic	• High sensitivity • High specificity • Analyses several markers simultaneously • More efficient than individual Western blots	• Does not reveal the heterogeneity of the cell population • Low throughput

		• Cost effective compared to individual Western blots • Does not require specialized equipment • Ideal for screening cell populations	
Real-time RT-PCR (Reverse Transcriptase Polymerase Chain Reaction)	Phenotypic	• Detects early changes in marker expression induced by differentiation • Analyses several markers simultaneously • High sensitivity and specificity	• Yields the average marker expression of a population • Does not reveal heterogeneity of the cell population

1.5.3 Cancer stem cell models

Two models were first proposed to explain the phenomenon of tumour heterogeneity: Firstly, the clonal evolution model, which suggests an alteration in individual cells via stochastic genetic [115] or epigenetic [116] alterations (see figure 1.5). In this model, cancer cells accumulate these alterations over time, and clones with selective growth advantage were selected. Indeed, extrinsic mechanisms such as different microenvironments within a tumour may confer phenotypic and functional differences upon cancer cells.

The second model, the CSC model, refers to the notion that a small population of cancer cells has the ability for self-renewal, and to maintain tumour growth, contrary to the rest of the cell populations (non-CSCs) (see Figure 1.5). In this model, the cells can also give rise to phenotypically different cells within a tumour with differentiation or "reprogramming" process [117]. However, this model does not address if the origin of cancer could be from healthy stem cells. Nevertheless, it points out that a hierarchical system exists in stem cells as much as in

healthy tissues, which means that if normal cells or stem cells can differentiate into phenotypically diverse progeny with limited proliferative potential, then CSCs can form phenotypically diverse non-tumorigenic cancer cells too. Epigenetic factors are thought to cause the loss of tumorigenic potential in cells that fail to contribute to disease progression. However, some reports *via* lineage-tracing experiments indicated that some cells are transformed stem cells that later act as CSCs; these transformed cells can originate from the skin, colon, and brain tumours [87], [118], [119]. There are also reports indicating that cellular dedifferentiation and reprogramming process can transform committed progenitors into CSCs [120]-[123].

To add a new level of complexity and heterogeneity within a tumour, there is a recently proposed third model (see figure 1.5)⁶; the plasticity model, postulates that cells can have dynamic interchangeability between CSCs and non-CSCs which is determined by internal and external factors, and not mutually exclusive to the prior models of clonal evolution or CSC [124]. Since not all cells have tumorigenic potential, to characterize malignant cells that potentially adhere to these models, it is essential to focus on the small population of stem-like cells among the tumorigenic cell's population

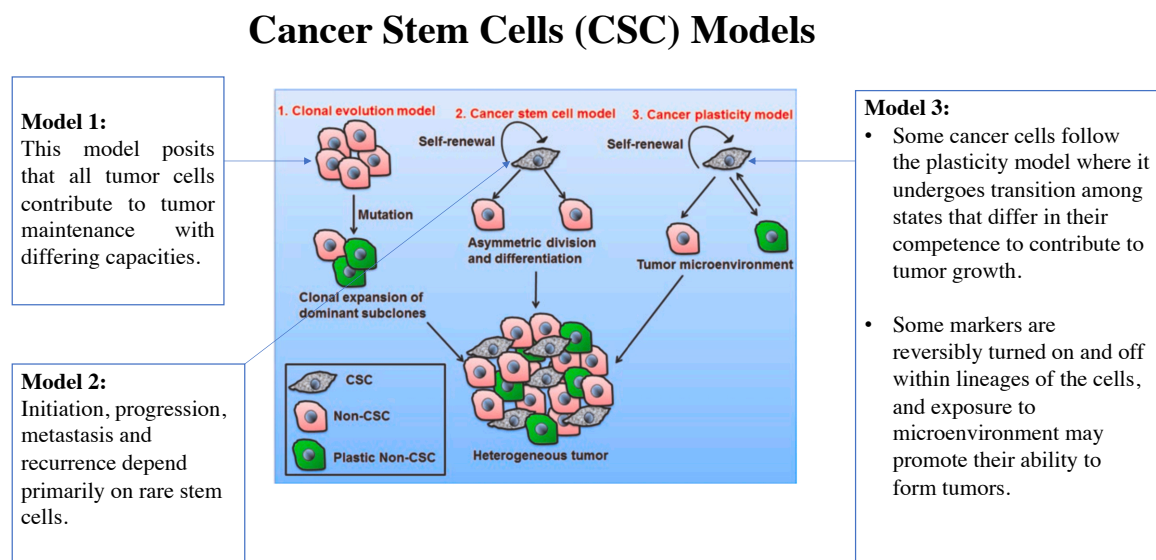


Figure 1.5: Three distinct models have been proposed to account for tumour growth and the heterogeneity within tumour. Model 1 (model 1 in your graph!) is called the clonal evolution model. This model posits that all tumour cells contribute to tumour maintenance with differing capacities. The intercellular variation is attributed to sub-clonal differences that result

⁶ Adapted from Naik, P. P., Panda, P. K., & Bhutia, S. K. (2017). Oral Cancer Stem Cells Microenvironment. *Advances in Experimental Medicine and Biology* (pp. 207–233).

from genetic or epigenetic changes during cancer development. Model 2 emphasized the modalities of cancer such as initiation, progression, metastasis and recurrence, which depend primarily on rare stem cells. This model suggests that tumours are highly hierarchical with a unique self-renewing population of cells at the top of the hierarchy. All other cells comprising the tumour bulk are derived from differentiated CSCs. An alternative view also exists. Lastly, Model 3 refers to the plasticity model, where some cancer cells undergo transition among states that differ in their competence to contribute to tumour growth. Some markers are reversibly turned on and off within lineages of the cells, and exposure to microenvironment may promote their ability to form tumour. These rare subpopulations in cancer cells exhibit resistance to a variety of therapeutics, reversibly forming sensitive or resistant progeny depending on the therapeutic approach.

1.5.4 Gastric cancer stem cells

Since the pathogenesis of gastric adenocarcinoma has been associated with abnormal patterns of gastric differentiation and with chronic gastric tissue injury, many investigations have focused on the response of stomach epithelial stem cells to inflammation. Chronic inflammation of the stomach lining and damage to the tissue, as induced by infection with *H. pylori*, affects differentiation and promotes metaplasia [6]. Dietary carcinogens, with or without the co-occurrence of *H. pylori* infection, are another risk factor for the mutation of the healthy stem cells in the corpus region, which can lead to metaplasia. In the case of gastric cancer stem cells (GCSC), there is an evidence of an association between stem cells and inflammation. In a mice study, Jean-Marie Houghton and colleagues demonstrated that bone marrow progenitor stems cells originating from bone marrow migrate to the stomach in their *H. pylori*-induced inflammation location where they consequently developed into GC [12]. This study proposed that adult stem cells can be transformed into cancerous cells, however, the causes driving the phenotypic changes are unclear. In Table 1.5, putative markers shared by gastric progenitor stem cell and GCSCs are compared (the progenitor markers' existence is exploited by CSCs to sustain their proliferative capacity within the stomach):

Table 1.5 Summary of putative gastric stem progenitor cells and cancer stem cell markers.

*n/t: not tested

Progenitor Markers	Location and function		References
	Normal stomach	Gastric cancer	
Villin promoter	Gland base, antrum of mouse stomach. Gives rise to all cell types (lineage tracing). Normally quiescent, multiplies in response to interferon gamma.	n/t	[125]
Lgr5	The base of the pyloric gland gives rise to all cell types (lineage tracing).	Luminal surface, tumour centre and invasion front (Immunostaining, 100 GC patients analysed)	[126] [127]
Sox9	Weak expression in the neck, or isthmus of the corpus, moderate expression in the neck, or isthmus of the pylorus (immunostaining)	Strong expression observed in GC (46 patients analysed by immunostaining)	[128]
Oct4	Isthmus region of the pit gland, pyloric antrum of the human stomach (immunostaining)	Increased expression in GC	[129]
CD44 (cell surface glycoprotein involved in cell-cell interactions,	Stable expression in lower glandular cells of the gastric antrum and rare expression in the corpus	Active expression in GC (isolated cells from cancer cell lines as well as mouse and human gastric samples produce spheroid colonies	[41] [130]

cell adhesion & migration)		and show tumorigenicity)	
CD24 (cell adhesion molecule, involved in Wnt signalling pathway, cell migration and self-renewal)	Side population of the small intestine (jejunum) CD24 population contains actively cycling cells and is enriched in Lgr5 expression	CD24 is associated with EGFR and supports EGF/EGFR signalling via RhoA (Ras homolog gene family, member A) in GC cells	[110]
CD44 ⁺ CD24 ⁺	n/t	Isolated cells from gastric tissues of patients produce spheroid colonies and show tumorigenicity	[131]
CD133 (encoded by PROM1 gene, and a glycoprotein)	The base of gastric glands, the antrum	Strong expression in GC, expressed on the luminal surface membrane of gland-forming cells	[94], [132], [133]
CD166 (transmembrane glycoprotein, important in cells aggressive characteristics)	n/t	Cytoplasmic and membranous CD166 positivity significantly correlated with nodal involvement and vascular invasion (142 GC patients tested with poorer outcome).	[95]

1.5.5 CSCs dynamic state and plasticity

To prove the CSCs dynamic plasticity, Roesch et al. [98] first demonstrated that sorted JARID1B histone demethylase positive cells in melanoma are highly tumorigenic. Knockdown of JARID1B negatively affects the proliferative potential of their progeny, suggesting JARID1B is a crucial regulator for tumorigenicity. However, JARID1B expression in JARID1B⁺ cells can still be positive regardless of cells tumorigenicity, suggesting that the cells do not follow a hierarchical cancer stem cell model [99]. The researchers also proposed that the stem-like cells are transient and can acquire stemness-like properties such as epithelial-to-mesenchymal transition (EMT). They also suggested that tumorigenicity is reversible depending on the tumour context. Another study, conducted by the Weinberg group, found that differentiated mammary epithelial cells do not adhere to the unidirectional hierarchical model but can convert to a stem-like state. This conversion follows a stochastic model *in vitro*, and oncogenic transformation enhances the conversion in which non-CSC can give rise to CSCs *in vitro* and *in vivo* [134]. Later, the same researchers also found that ZEB1 (a well-characterized EMT transcription factor) controls stimuli-mediated plasticity in basal cells (which are not luminal) and that non-CSC respond to micro-environmental stimuli in order to be converted to a CSC-like state [135].

The phenomenon of phenotypic change can be possibly explained by potential reprogramming to further validate the dynamic stemness model. Suva and colleagues [136] found that combined induction of stemness *POU3F2* (*OCT3*) + *SOX2* + *SALL2* + *OLIG2* transcription factors in differentiated glioblastoma cells produced cells capable of forming a tumour in 100% of animals they studied [137]. They induced reprogramming of differentiated cells into stem-like cells when stemness-transcription factor (TF) binds and activates CSC-specific regulatory elements in the cells. Similarly, a study of colorectal cells found that the induction of *OCT3/4*, *SOX2*, and *KLF4* can enhance CSC properties of the cells [138]. It is also important to point out that specific micro-environmental signals arising in the tumour may induce CSC function and plasticity [139]. A question remains: if stemness is an acquired characteristic of CSCs, can external factors or environmental agents alter a cell's stemness phenotype to acquire a dynamic plasticity?

1.6 Cells phenotypic change and plasticity

In response to an environmental stimulus, microbes such as yeast and bacteria are known to adopt specific phenotypic response, and in some cases, after the removal of the stimulus, these phenotypes are preserved. In the event of unfavourable environmental changes, the preserved phenotypes can be useful for survival [140].

Under certain physiological conditions, mammalian cells can also undergo dynamic and coordinated cellular response to internal or external stimuli during cellular development. Some cells can hijack this ability to adapt to unfavourable conditions, potentially leading to the formation of tumour cells. For example, by altering fundamental mechanisms, some malignant cells can be reprogrammed to exploit energy metabolism and evade destruction by the immune system. These changes at the genetic level are manifested in various phenotypic characteristics, which contributes to the complexities of this disease. This is one of the hallmarks of cancer [1], [141].

1.6.1 How does the cell's plasticity affect phenotypic change?

In response to some physiological stresses such as inflammation, injury, or senescence, many non-transformed differentiated cells can attain some form of plasticity. The term 'plasticity' means mouldable or alterable⁷ but in cancer biology, it is identified in a phenomenon biologists refer to as cellular plasticity [142]-[144]. Phenotypic plasticity model describes plasticity as a means that allows for reversible change within tumorigenic cells, which is also a driving force of a phenotypic heterogeneity [97].

However, the term cellular plasticity can also cover broad examples. Several studies have shown that compensatory and adaptive mechanisms are available to stem cells within different population pools [145]-[147]. For example, a study in which ablated Lgr5-expression cells in mice *via* Lgr5 knock-down with diphtheria toxin receptor (DTR) gene showed increased production of Bmi1-expressing progeny cells in return indicated that the loss of Lgr5-expressing cells is compensated by Bmi1-expressing stem cells [145]-[147]. In the event of physiological stress or organ injury, adult cells can also respond by de-differentiating or trans-differentiating [148], [149]. In cancer, trans-differentiation events may occur before the

⁷ <https://www.merriam-webster.com/dictionary/plasticity>

oncogenic steps in intrahepatic cholangiocarcinoma (ICC, a cancer of the bile ducts of the liver). Although it is not entirely clear whether the plasticity event occurs before or after the oncogenic events, it is tempting to speculate that underneath cellular plasticity, aberrant changes in gene expression and chromatin structure could lead to abnormality of tumour suppressor gene repression and expression of oncogenes. Oncogene driver mutation or changes in chromatin factor are known to contribute to cellular reprogramming and promote cell malignant transformation [137]. Thus, it has been a popular hypothesis that cell plasticity is an important characteristic activated in cancer [142]-[144], [150]-[152].

Reprogrammed cells that form a tumour undergo several metabolic changes, especially in manipulating and modifying main metabolic pathways to cope with the increased demand for energy and nutrients due to increased proliferation [153]. The termed “Warburg Effect” is synonym when discussing the altered metabolic phenotype of cancer cells [154]. Therefore, to cope with these conditions, cells undergo morphological changes, often leading to provide increased invasiveness and angiogenic capabilities [141]. Thus, these studies suggest that plasticity is an intrinsic ability of any cells which leads to various phenotypic change in order to cope with biological demands or circumstances. A tumour cell's ability to alter the biological rules in an organism signifies an essential challenge for oncological research.

1.7 The Paradox of the cancer hallmarks

In the landmark papers of cancer hallmarks I [141] and II [1], Hanahan and Weinberg described that when a cell acquires or manifests 8 essential alterations in its physiology (sustained angiogenesis, insensitivity to anti-growth signals, evasion of apoptosis, self-sufficiency in growth signals, limitless replicative potential, tissue invasion and metastasis, dysregulated metabolism and evading the immune system), it would result in malignant growth (refer to Figure 1.6). However, it is intriguing to ask: should the hallmarks be malignant and not benign features; it could be argued that sustained angiogenesis is a feature of both benign and malignant tumours [181]. For example, in order to survive, a mass of tissue larger than a few milligrams would require sustained angiogenesis [182], [183]. Therefore, tumour angiogenesis is not an exclusive feature of malignancy. In addition, without evasion of cell death and insensitivity to anti-growth signals [141], it is unlikely that so many abnormal cells could accumulate and survive the control mechanism of normal tissue homeostasis process. For example, an antigrowth and tumour suppressor protein, *Rb1*, which must be inactivated to

develop a tumour [187]-[189], is deficient in both retinoma (a benign eye tumour) and retinoblastoma (a malignant eye tumour) . In addition to that, anti-apoptotic protein *Bcl-2* is linked with better patient survival and outcomes in breast [187]-[189], colon [190]-[192], and lung cancer [193]-[195] patients. Contrariwise, other apoptosis-inducing protein such as *Bax* [196], [197], *CD95* [229], *Caspase-3* [230] were found to encourage carcinogenesis [199], [200]. Nevertheless, one of the hallmarks – tissue invasion and metastasis – according to some opinions [155]-[157], is the exceptional feature of a malignant tumour, whereas benign tumours do not invade or metastasize in general (with an exception in the case of benign tumour cells in tuberous sclerosis complex [201]).

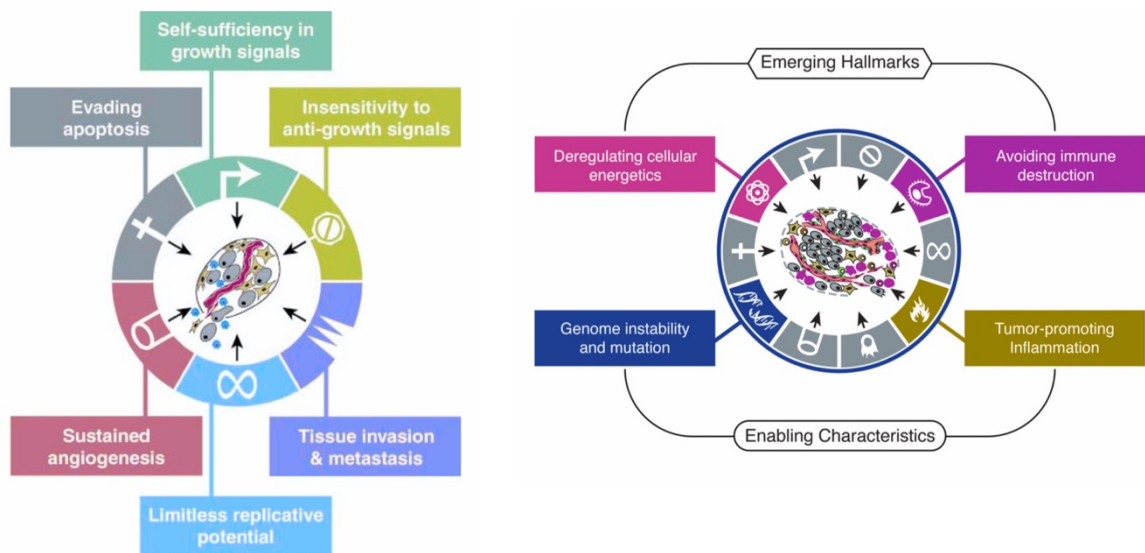


Figure 1.6: Cancer hallmarks. (Adapted from [1] and [161]) The left figure showed the initial hallmarks proposed by Hanahan and Weinberg, and the right figure is latest addition to the existing hallmarks.

1.7.1 Tumorigenic potential and the fate of cancer cells

In addressing the opinion that CSCs are the main force behind malignant tumour, currently, many studies do not view the cancer stem cell model as a “potential” option but rather assume that this is the “fate” of the cells. “Potential describes what cells are capable of doing under permissive conditions, whereas fate describes what they do in a specific circumstance” [158]. For example, some cells with high tumorigenicity potential are killed by immune cells, or by therapy because they failed to thrive and drive the tumour growth in its permissive native environment. However, some cells (e.g. stromal cells) with low tumorigenicity potential, upon transplantation, manage to form tumours due to the absence of a growth restrictor in the native

environment [137], [207]-[210] (refer Figure 1.7). On the other hand, breast cancer tends to metastasize to distant organs (which is responsible for over 90% of breast cancer-related deaths) in lung [137], [211], liver [212], [213], bone [214], [215], and brain [216] through local, lymphatic and vascular invasion; its growth and survival are aided by fortifying the target organ's surrounding microenvironment [159]. However, how these processes occur still remains unclear [159], [160].

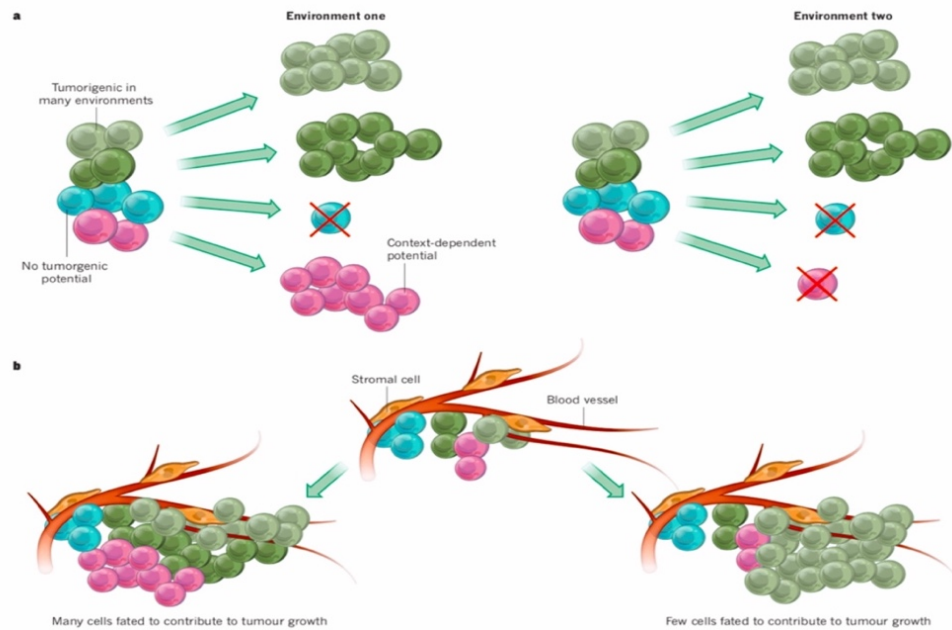


Figure 1.7: Cancer cell's fate versus potential. (A) Golden standard of accessing the potential of cancer cells to form tumours is to conduct transplantation assays. The pretext of the transplantation assay: in environment one, cells that can usually form a tumour may not form a tumour in environment two; such that studies must not underestimate the spectrum of cells with tumorigenic potential, and an experiment needs to be conducted under permissive condition. (B) To access the actual fate of tumour cells, the use of lineage tracing or fate-mapping assays is essential, often in the tumour native environment. While potential measures what cells can do under certain conditions, fate measures what cells "actually" do in particular situation. This is important because some potential tumorigenic cells, in non-permissive environment, do not form a tumour, for example, due to the elimination of immune effector cells. However, the critical question remains which tumorigenic cells - many (left) or few (right) - has the potential to contribute to tumour growth. Image adapted from [142].

Thus, – the classical view coined by Fearon and Vogelstein in the 1990s explaining how tumour cells get metastatic capacity is that the ultimate malignant transformation is a result of accumulating genomic alterations [220]. However, should a metastatic phenotype be the only advantageous criteria of a primary tumour? An alternative model proposed that this phenotype was determined by the coincidental acquisition of mutant alleles during early carcinogenesis, which contributed to malignancy advantage, which made metastasis an inherent phenotype of

a malignant tumour [221]. If the accidental acquisition of mutant alleles results in metastasis, why do some malignancies always metastasize such as breast carcinoma [222], yet some others such as basal cell carcinoma [161], rarely do but actively invade nearby cells?

The answer could potentially lie in the stem-like population within the tumour, which has the inherent metastatic phenotype. Epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) are *bona fide* processes essential to endow the cells with stem cell characteristics in embryogenesis and metastasis (EMT), and differentiation and de-differentiation (or reprogramming; MET) [224], [225]. Tumour cells exhibiting EMT tend to have the highest plasticity and migratory ability to advantageously settle in secondary sites [150]. Disseminating tumour cells need to undergo MET, the reversal of EMT, to manifest metastasis after migration [162]. Thus, this possibly explains the co-expression of epithelial markers and EMT-inducing transcription markers with CSC stemness markers in colon CTC (circulating tumour cells) cancer in cultures [28].

1.8 Epithelial–Mesenchymal Transition (EMT) vs. Mesenchymal–Epithelial Transition (MET)

EMT is the process of the transformation of epithelial cells into mesenchymal cells, whereas, the reversal process of EMT, MET is a biological process that transforms the spindle-shaped mesenchymal cells into the apical-basal polarised epithelial cells. In the 1980s, Betty Hay first described this process while observing chick embryo developing tissue and organs [229]..

In general, epithelial cells appeared as adherent cells with apical-basal polarity. The cells communicate with each another through intercellular junctions, via membrane protein and integrins interaction [230]. In EMT events, epithelial cells lose their apical-basal polarity and their cell-cell junctions, and acquire a front-rear polarity like a mesenchymal cell for increased motility [246], [248]. They also degrade their extracellular matrix protein to enable invasive behaviour [220]. Destabilising intercellular adherent junctions by downregulation of *E-Cadherin* is one of the hallmarks of EMT [93], [231] (refer to Figure 1.8 for characteristics of EMT-cell types). The master regulators of these event cascades are EMT-transcription factors (EMT-TFs) such as *Snail*, *TWIST*, and zinc-finger E-box-binding (*ZEB*), which play a central role in regulating the changes in gene expression that potentially activate or repress epithelial or mesenchymal phenotypes (Figure 1.9) [135], [163].

Epithelial cells 	• Cell-cell adhesion • Apical-basal polarity • Epithelial markers: <i>E-Cadherin</i>, <i>Cytokeratin</i>, <i>Laminins</i>, <i>Collagen type IV</i> etc. • <i>Cortical actin</i> (modulate the cell surface properties and membrane behaviour)
Partial EMT 	• Cell contact dissolution, actin reorganization • Stemness markers: <i>ALDH1</i>, <i>ABCG2</i>, <i>OCT3/4</i>, <i>SOX2</i>, <i>NANOG</i>, etc.
Mesenchymal Cells 	• Cell individualization • Front-rear polarity • Mesenchymal markers: <i>N-Cadherin</i>, <i>Vimentin</i>, <i>Fibronectin</i>, etc. • Actin stress fibre (enables contractility and cell motility)

Figure 1.8: Characteristics of epithelial cells, partial-EMT cells, and mesenchymal cells involved in the EMT/MET process.

As a fundamental biological process, EMT/MET is involved in various stages of development: from embryonic stem cells differentiation and induced pluripotency, to wound healing and cancer progression, especially in the early conversion of tumours into invasive malignancies. Several studies indicated that overexpression of EMT-TFs contributed to tumour formation [135], while induction of EMT generates population with CSC-like properties [151], [164]. Arguably, many primary epithelial tumours grow in differentiated forms, owning their basic phenotype and morphology of its original cells, often without any evidence of EMT. However, these primary tumours do metastasise by acquiring the mesenchymal phenotype to begin the EMT process [233]. Naturally, to achieve this, acquiring ‘stemness’ would be the priority step, so that phenotypic change can take place in the tumour, often by regulating embryonic pathway such as Wnt pathway [92], which is abnormally activated in cancer [165], [166]. Therefore, although EMT and stemness are both features of CSC-like cells, yet these processes are not always directly coupled. For example, low-level expression of *TWIST* is important for inducing stemness in mouse skin cancer cells, however high-level expression of *TWIST* induces EMT [212], [236]. In contrast, expression of *Snail* in bladder and prostate cancer induces EMT, yet it suppresses stemness [167]. Thus, there is a “missing-phase” between a tumour initiating EMT process and one acquiring stemness. The EMT/MET process only represents the two different states, namely epithelial or mesenchymal; however, it may generate a transitional state called partial-EMT [1], [143]. Depending on the signalling pathway and tissue location, some studies found that the partial-EMT in breast cancer tumour [241], [257]-[261], ovarian cancer [237], cell lines of lung and renal carcinoma [238]-[241], mouse pancreatic and prostate cancer [242], exhibits the integrity of epithelial phenotype, yet it expresses mesenchymal markers along with its plasticity response (refer Figure 1.9). Notably, gastric cancer (GC) cell lines ⁸, SNU-539 and SNU-1077 cells showed positivity for both epithelial and mesenchymal markers via antigen staining [31].

⁸ In another study of a series of human GC cell lines, low expression of the epithelial marker was detected in primary tumour (AGS and SNU-1), whereas increased level of expression was detected from cell lines derived from distant metastasis (MKN-7, MKN-45, NCI-N87, KATO-III, and SNU-5). (Burgermeister, E., Xing, X., Röcken, C., Juhasz, M., Chen, J., Hiber, M., et al. (2007). Differential Expression and Function of Caveolin-1 in Human Gastric Cancer Progression. *Cancer Research*, 67(18), 8519–8526.)

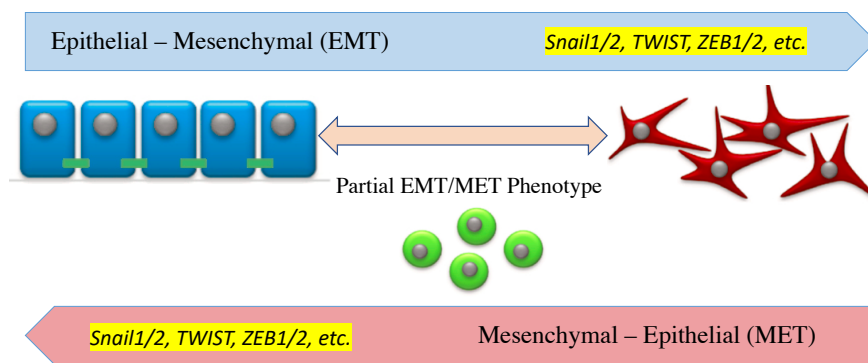


Figure 1.9: Blue arrow to the right signifies EMT process, whereas the red arrow to the left signifies MET process. Both processes utilize the main EMT-Transcription Factors such as SNAIL, TWIST, and ZEB, which either repress or activate key genes such as β -catenin through specialised pathway such as Wnt. This process also generates cells at the intermediate state called Partial EMT/MET with stemness phenotype.

Interestingly, all tumour subtypes of breast cancer cells with co-expression of epithelial and mesenchymal genes promote stemness and form a mammosphere (breast cancer CSCs tend to form mammosphere) is associated with the most unfortunate outcome in luminal and basal breast cancer patients [245], [246]. Partial EMT cells in mouse prostate cancer showed a more significant potential to initiate tumour and sphere formation [167]. Therefore, it is possible that stemness can be closely connected to the intermediate state of EMT.

1.8.1 Stemness potential in CSCs

Despite strong evidence supporting a stem cell role as a driving force in cancer, there are several important caveats to be considered. The lack of *bona fide* CSC markers, other than only three or four surface markers used in transplanted CSCs to recapitulate the heterogeneity of tumours, remains an obstacle to studying the capacity of cancer cells to acquire the stem cell phenotype. It also remains undetermined and perhaps underestimated whether phenotypic and genetic heterogeneity within primary tumours is the main contributor to tumorigenicity in cancer. However, this can be examined by studying subpopulations individually within a cell line (derived from the primary tumour), or within a tumour [99], [168].

Therefore, it is critical to determine if the subpopulation that is regarded as CSCs has the "potential" to contribute to cancer progression and make the tumour malignant. If we assume that most cancer cells have this potential to proliferate and metastasize significantly, then for a successful cancer treatment, all cancer cells must be eliminated. Therefore traditionally, most therapies aim to eliminate as many cancer cells as possible. However, this does not solve the

problem of possible post-cancer treatment relapse. Contrary to this, the CSC model proposes that there are small sub-populations of CSC within tumours that drive the growth and progression of many cancers [114], [169]. However, the paradox of this theory is that CSC marker expression indicates that tumorigenic cells (TC) and non-tumorigenic cells (NTC) are intrinsically different, yet there are no clear morphological distinctions between them [91].

Interestingly, Al-Hajj et al. also found that in breast cancer not all TC subpopulations can give rise to a tumour but these TCs can also generate phenotypically diverse NTC cells [91]. Their observation suggested that the differences between TCs and NTCs may lie in epigenetic differences because it is improbable that only rare cancer (*i.e.* TC) cells have the necessary genotype permitting extensive proliferation. However, in another study, increased proliferative potential, enhanced tumorigenicity and poor breast cancer survival was found to be linked with EMT markers and stemness expressions [170]. Therefore, depending on the cell-line studied, it is possible that stemness markers and stemness to EMT markers are intertwined: both could potentially form a stronger CSC paradigm that would possibly explain how some cells have the ‘potential’, while some do not. Thus, it is important to take into consideration the “stemness potential and EMT phenotypes” relationship by inducing its phenotypic changes in a controlled environment.

1.8.2 Exploiting cell’s plasticity: Induction of phenotypic change

According to the third CSC model, the concept of plasticity of the bidirectional conversion between non-tumorigenic and tumorigenic cells [171], or stem and non-stem cells [98], suggests that the heterogeneous cancer cell population is highly adaptable, capable of plasticity and transient evolution. The acquisition of stemness by non-stem cells is particularly crucial as stemness play a principal role in the early embryogenesis and development process, in which motile mesenchymal cells are derived from transdifferentiated epithelial cells [254]. The conversion process into cells with stemness properties (akin to mesenchymal cells) involves modifying the epithelial cell cytoskeleton, reprogramming signalling patterns, and finally changes in gene expression. This process can contribute to migratory and increased motility in order to invade nearby tissues [164] (Figure 1.10) With the understanding of this concept, a research group from Kyoto, Japan, induced stemness properties in colon cancer cells with defined factors (*OCT3/4*, *SOX2*, and *KLF4*). The induced cells exhibited enhanced stemness and CSC properties (in regards to marker gene expression, sphere formation, increased chemoresistance), and, most importantly, the transduced cells showed increased tumorigenicity [138].

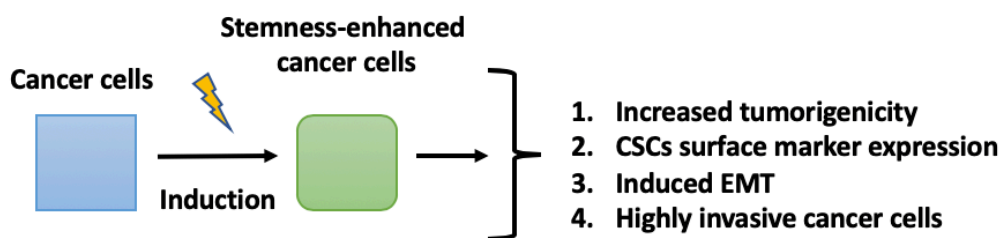


Figure 1.10: A summarized process of the induction of CSC properties in a cancer cell

1.8.3 Inducing agent: Benzo[a]pyrene, the environmental carcinogen

There is an inevitable link between the environmental and genetic origins of cancer. It is well established that mutation in four groups of genes: Oncogenes, Tumour suppressor genes, Apoptosis genes and DNA repairing genes, individually, or collectively, can lead to human cancers. Several models were proposed to explain the causes of cancer, mainly by Hanahan and Weinberg (the cancer hallmarks model) [1], [141], and Vogelstein and Kinzler, who emphasise that cancer is a disease of damaged DNA, resulting in the transformation of healthy cells through the multistep process of genetic mutation into cancerous cells [269].

It is well known that cigarette smoke from tobacco is a rich source of carcinogens that are harmful to healthy cells [172]-[175]. One of the thousands of carcinogens present in tobacco smoke is benzo[a]pyrene. Benzo[a]pyrene (BaP) is one of the best studied polycyclic aromatic hydrocarbons (PAH) classified as group 1 human carcinogen by the International Agency for Research on Cancer (IARC) [176]. Being classified as group 1 means that it is a confirmed human carcinogen [174]. When subjected to benzo[a]pyrene (BAP) [177] or other organic molecules such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin, TCDD) [178], the ligand (e.g. BaP/TCDD) binds to Aryl hydrocarbon Receptor (*AhR*) and forms an *AhR* receptor complex (*AhR*+ligands). This complex translocates to the nucleus and forms a heterodimer with Aryl hydrocarbon Nuclear Translocator (*ARNT*) [179], [180] to recognize a XRE binding site (Xenobiotic response element) [181], located at the upstream regulatory region of gene targets such as cytochrome P450 (e.g. *CYP1A1* and *CYP1B1*) [180], [182]. BaP metabolism by CYPs and epoxide hydrolase results in mutagenic and carcinogenic metabolites such as B[a]p-7,8-dihydrodiol-9,10-epoxide (BPDE) (refer Figure 1.11). BPDE can covalently bind DNA guanine residues to form pre-mutagenic adducts, leading to genomic mutation, which occurs

at critical gene targets (*e.g.* oncogenes) and can transform a normal cell into a cancer cell [183], [184]. Thus, with exposure by ingestion, for example, BaP can induce carcinogenesis in the stomach and promote cell proliferation and metastasis through *AhR* activation [177], [185]. Notably, a similar study showed that the BaP effect was lost in mice that lack the *AhR* receptor [186].

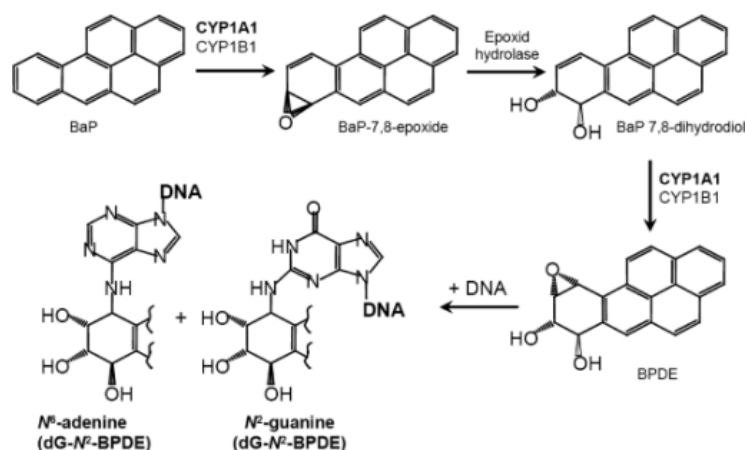


Figure 1.11: The 3-step activation process of metabolic activation of Benzo[a]pyrene that leads to DNA adduct formation by CYP family and epoxide hydrolase that leads to the formation of the ultimate carcinogen - Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE).

Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide is the carcinogenic product of three enzymatic reactions:

- Benzo[a]pyrene is first oxidized by cytochrome P450 enzymes to form a variety of products, including (+) benzo[a]pyrene-7,8-epoxide.
- This product is metabolized by epoxide hydrolase, opening up the epoxide ring to yield (-) benzo[a]pyrene-7,8-dihydrodiol.
- The ultimate carcinogen is formed after another reaction with cytochrome P450 1 enzymes to yield the (+) benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide.
- It is this diol epoxide that covalently binds to DNA that leads to mutation.

BaP's ability as a potent carcinogen has been well documented; however, not much is known on its effect on the stemness population. In mesenchymal stem cells (MSC), BaP exposure hinders the cells' self-renewal and differentiation capabilities [187]. Similar disruption to stemness activity was observed in placenta-derived MSCs [218], [266]. However, BaP induces profound effects in tumorigenic cells, for example in lung cancer, in which BaP exposure upregulates stemness marker expression (*ALDH1*) in BEAS-2B cell line [81], and causes various alterations in gene expressions in A549 lung cancer cell line in a microarray study [268]. In gastric cancer cell line, increased proliferation, migratory and invasion capabilities

were observed when cells were exposed to BaP *via* AhR activation [177]. However, at the point of writing, nothing is known whether the induced malignancy potential by chemical carcinogen and ‘enhanced’ stemness phenotypes can be associated with any subpopulation of cancer.

1.8.3.1 The role of aryl hydrocarbon receptor (AhR)

The Aryl hydrocarbon receptor (*AhR*) is an essential gene that encodes a protein that plays a significant role in many biological functions [175], [188]-[192]. AhR protein is responsible for a ligand-activated transcription factor that binds to exogenous ligands and performs essential functions in xenobiotic-induced metabolism, toxicity and carcinogenicity [186]. Unliganded AhR that resides in the cytoplasm binds to chaperone molecules to form a complex. Upon binding to a ligand, (for example benzo[a]pyrene (BaP) [177] or 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin, TCDD)) [178], the receptor complex translocate to the nucleus and forms a heterodimer with Aryl hydrocarbon Nuclear Translocator (*ARNT*) [179], [180]. Here, it will recognize a XRE binding site (Xenobiotic response element) [181], located at the upstream regulatory region of gene targets, mainly the cytochrome P450s such as *CYP1A1* and *CYP1B1* (refer Figure 1.12). It is also worth noting that *AhR* can be activated by endogenous ligands that could lead to cell-type specific *AhR*-dependent effects [193].

Metabolic activation of BaP that leads to DNA damage

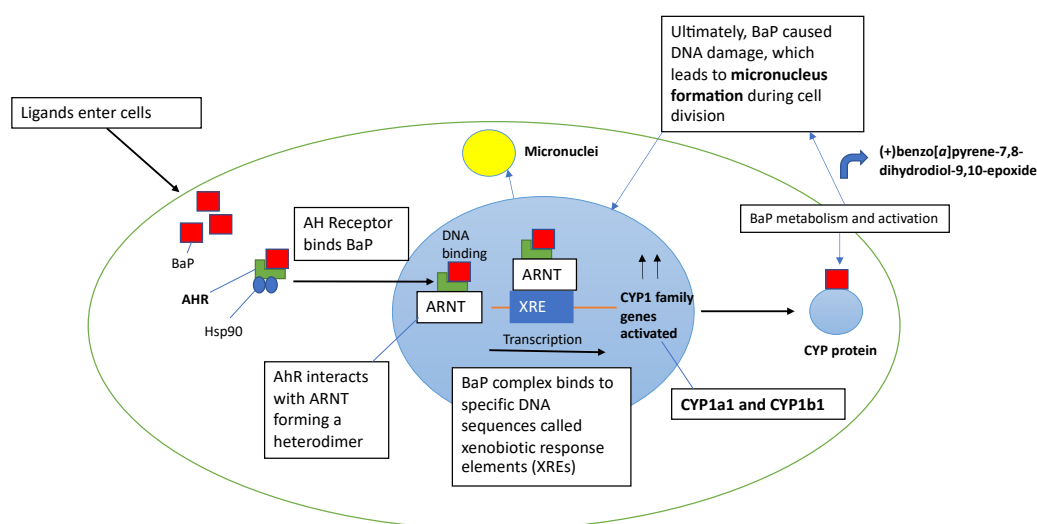


Figure 1.12: Metabolic activation of BaP that leads to DNA damage in a cell.

Additionally, a recent study found that *SNAIL* or *Snail* (snail family zinc finger 1) family could act as a direct transcriptional target for specific ligands, for example, TCDD, to increase mesenchymal markers⁹ [194], which may potentially lead to epithelial-mesenchymal transition (EMT), and thus induce some level of plasticity in the cell [195]. *CTNNB1* (along with *MYC*) is an essential gene in the *WNT* signalling pathway [196], which codes for a protein that is part of a complex involved in adherens junctions (AJs). The CTNNB1 protein regulates cell proliferation, cell growth, adhesion between cells [166], [197], and cell cycle [166], [198].

MYC (consisting of three related genes: *C-MYC*, *I-MYC*, and *N-MYC*), an important proto-oncogene that encodes for a transcription factor, is consistently expressed in many cancers [300]. In gastric cancer, BaP induces proliferation and metastasis by *AhR* and ERK-dependent induction of MMP9 and *C-MYC* [177]. Its close association with *CTNNB1* also means that it is likely to be involved in EMT: a study showed that an overexpression of *MYC* can induce EMT in mammary cells [288]. Interestingly, AhR interacts with several signalling pathways that govern cell cycle, proliferation, cell adhesion and cell migration [199]. Thus, it is possible that *AhR* has a significant role in the gene regulation of *Snail* and *CTNNB1* and the stemness genes. Thus, recent research in *AhR* biology suggests that the AhR complex is involved in various biological functions from cell differentiation, cell pluripotency, stemness, within immune, cardiovascular to reproductive systems [191], [199].

⁹ Mesenchymal markers also include expression of cell surface markers including CD44, and CD166, amongst others [46].

1.9 Hypotheses:

1. Stem cell-like population can be identified and isolated in gastric cancer (GC) cell lines.
2. Environmental chemicals phenotypically alter gastric cancer cell lines to promote a stem-like phenotype.

1.9.1 Aims of the study

- **Chapter 2:** Identify and isolate stem cell-like subpopulations from gastric cancer cell lines.
- **Chapter 3:** Phenotypically characterise the sorted SNU-1 population with regards to stemness gene expression, and the expressions of essential genes involved in xenobiotic response and EMT.
- **Chapter 4:** Investigate whether the carcinogen exposure correlates with the changes of stemness markers within gastric cancer cells.
- **Chapter 5:** Determine the effect of BaP treatment on gastric cancer cell subpopulations, on the expression of stemness markers and on DNA damage.

2 Characterization of Gastric Cancer Cell Lines

2.1 Introduction

Gastric cancer (GC) is the 5th most commonly diagnosed type of cancer¹⁰, moreover, it is the leading cause of cancer-related mortality in 2018 [5]. Although *H. pylori* infection is the main factor contributing to stomach cancer, other factors such as dietary components (food preserved by salt, or low fruit intake), alcohol ingestion and cigarette smoking are also influential contributing factors [200].

To assist scientists and clinicians worldwide in characterising and diagnosing GC, World Health Organization (WHO) classified the digestive tumour into five classifications: poorly cohesive carcinoma (with or without signet ring cells), mixed carcinoma, papillary, tubular and mucinous adenocarcinoma [201]. On the other hand, the classical Lauren P. classification is less complicated, and it described two basic types of GC – intestinal and diffuse [9]. Because cancer is a genetic disease, GC might contain various prominent genetic alterations that lead to aberrant gene mutation, amplification and epigenetic alterations such as at the promoter DNA CpG islands [202]. These two pathological types (diffuse and intestinal) also have very distinct molecular profiles [202]. The gastric NCI-N87 cell line was classified as Lauren-intestinal gastric type, whereas SNU-1 and KATO-III cell line were classified as Lauren-diffuse gastric type DT [44].

At present, treatment of GC remains a challenge where surgery is the only means for early-stage (I & II) and – with the addition of neoadjuvant chemotherapy – for stage III disease GC patients [203]. However, many patients who underwent surgery later develop local or distant recurrences, which lowers their survival rate [204]. The cancer stem cell (CSC) theory suggests that there is a subset of cells that have the self-renewal and cell differentiation ability similar to healthy stem cells [114]. Studies have also indicated that CSCs are more resistant to chemotherapy [44] than non-CSCs, and can cause relapse [169], [205]. Therefore, isolating and characterizing CSC population have been gaining popularity within the scientific community for almost a decade now.

¹⁰ <https://www.wcrf.org/dietandcancer/cancer-trends/worldwide-cancer-data> (Accessed May 2018)

One of the methods of characterisation and identification of the subset of CSCs is by studying the expression of specific cell surface markers of the side populations within the whole population of tumour or cell line. Takaishi *et al.* explored various CSC-potential markers such as CD24, CD44, CD166, and CD133 in seven gastric cancer cell lines and found that CD44⁺ subpopulation has greater tumour initiating capacity in mice as well as greater resistance to chemotherapy [41]. Also, a sizeable CD44⁺ population was found in various gastric cell lines, and the sub-population of CD44⁺ has increased expression of stemness transcription factor of *SOX2*, and *NANOG* [44]. Other markers such as CD24⁺ cells from primary pancreatic adenocarcinoma [206], and CD133⁺ cells from gastric cancer cell lines also showed potential to initiate tumour when injected in mice, had stem cell self-renewability properties and were able to produce differentiated progeny. All of these characteristics are CSC hallmarks ([114], [207], [208])

Currently, it is understood that cancer cells are heterogeneous with respect to proliferation and differentiation [207], which makes characterising CSC-like population more difficult. In addition, there are several conflicting studies indicating that some CSCs markers such as CD44 and CD133 from primary human GC may not exhibit tumour-initiating properties in xenograft transplantation experiments nor expressed stem-like properties in immunocompromised mice [105]. Similarly, in colorectal cancer cell lines – CD24⁺, CD44⁺, CD166⁺, and CD133⁺ – phenotypes fail to identify CSC population and its stem-like features [209]. Thus, in this chapter, I characterised the GC cell lines (SNU-1, KATO-III, and NCI-N87), to identify the potential 'stem-like' cells before investigating the phenotypic change that these cells may undergo with any external induction.

2.1.1. Hypotheses:

- Subpopulations with the expression of CSC markers of interest can be identified from gastric cancer cell lines.
- Genes related to pluripotency (*e.g. SOX2, OCT3/4, NANOG*) can be identified in the cells expressing the CSC markers.
- The pluripotent markers expressed in these cell lines are temporally regulated.

2.2 Materials and Methods

2.2.1 Cell Culture

Human gastric cancer cell lines (SNU-1, NCI-N87, KATO III) were purchased from American Type Culture Collection (ATCC) and cultured as recommended by ATCC. SNU-1 and NCI-N87 were cultured in ATCC RPMI-1640 Medium (ATCC® 30-2001™) with 10% foetal bovine serum (FBS), whereas KATO III was cultured in ATCC-formulated Iscove's Modified Dulbecco's Medium (Catalogue No. 30- 2005) in 20% FBS. Both were cultured in T75 flasks. Two different passages were taken for the flow cytometry analysis, early passage (70-90% confluence) and cells from the same flask that were left to grow for several more passages before harvest for late passage (confluent 70-90%). The early passage in the experiments was referred to as SNU-1, KATO-III and NCI-N87 at passage 10 (P10), whereas the late passage was referred to as SNU-1, KATO III and NCI-N87 at passage 44 (P44).

2.2.2 Flow Cytometry Analysis

For surface marker analysis by flow cytometry, cells were grown to 70%-90% confluency in T75 flasks (Appleton Woods, Cat. No. 430641). For suspension cell line, SNU-1, the medium was removed into a 50 ml tube. For KATO-III and NCI-N87, the media were removed and washed once with 20 ml sterile phosphate-buffered saline (PBS), and then the cells were dissociated from plates using 1 ml of Trypsin-EDTA (Invitrogen; 1:10 dilution of stock, at 37°C) and left in an incubator for 5-7 minutes to detach. For firmly attached adherent cells (NCI-N87, KATO III), an additional 5-minute incubation was required for the attached cells to detach fully. The cells were transferred to a 15 ml tube, and 2 ml of the respective media suitable for each cell line (twice the volume of the Trypsin used) were added to deactivate the enzymatic reaction of the Trypsin. The cells were centrifuged at 400×g for 10 minutes, supernatants were discarded, and then the cells were resuspended in 1 ml of the RPMI or Iscove's medium. Cell counts were performed using an automated cell counter machine (Moxi from ORFLO Technologies), and 10⁶ cells were added into each FACS tube and incubated on ice for 15 minutes. New medium (1 ml) was added to as many tubes as required and centrifuged for 500×g for 5 minutes. 30µl of PBA (1% Bovine serum albumin dissolved in phosphate buffered solution) containing 3 µl of FcR Blocking reagents (Miltenyi Biotec) were added to each tube containing the cells to reduce non-specific binding of the antibody. PBA was used

because it helps to stabilize the cell membrane and prevents antibodies from sinking into the membrane.

Next, the supernatants were discarded, and 10 µl of an antibody of each CD markers (refer table 2.1 for concentrations) dissolved in 100 µl of staining buffer (aqueous buffered solution containing foetal bovine serum and 1% sodium azide) (Becton Dickinson, USA) were added to 10⁶ cells pellet in each respective tube labelled with the corresponding CD markers being used and then incubated for 30 minutes on ice. It is worth noting that antibodies were titrated to determine optimal antibody concentration for live-cell labelling to achieve the best discrimination between positive and negative cells, while keeping nonspecific antibody binding to a minimum, according to the protocol's recommendation [210]. Finally, 500 µl of PBA added to each tube, and samples were centrifuged for 400×g for 15 minutes, and the supernatant was discarded to remove excess antibodies. Cell pellets were resuspended in 1 ml of PBA and were examined with BD FACS Fortessa flow machine (Becton Dickinson, USA). The results were analysed using-FlowJo (version 10.1r1) software on Mac OS X El-Capitan (version 10.11.5) Operating System.

Table 2.1 CD markers used in the experiments:

CD Markers	Supplier	Supplier items code	Concentration (per 10µl)
APC Mouse Anti-Human CD44	Becton Dickinson	559942	0.25ug/µl
FITC Mouse Anti-Human CD24	Becton Dickinson	555427	0.2ug/µl
PE Mouse Anti-Human CD166	Becton Dickinson	559263	0.0125ug/µl
PE-Vio770-CD133/1, human	Miltenyi Biotech	130-101-676	0.014ug/µl

The sample set and preparation were the same for both passages of the various cell lines. Fluorescence Minus One (FMO) Control was used in this experiment. The FMO control is used to correctly interpret flow cytometry data, especially in the case in which the population of interest is meagre, FMO acts as a reasonable quality control. It is used to identify and gate cells

in the context of data spread due to the multiple fluorochromes in each panel. (Refer to Figure 2.1)

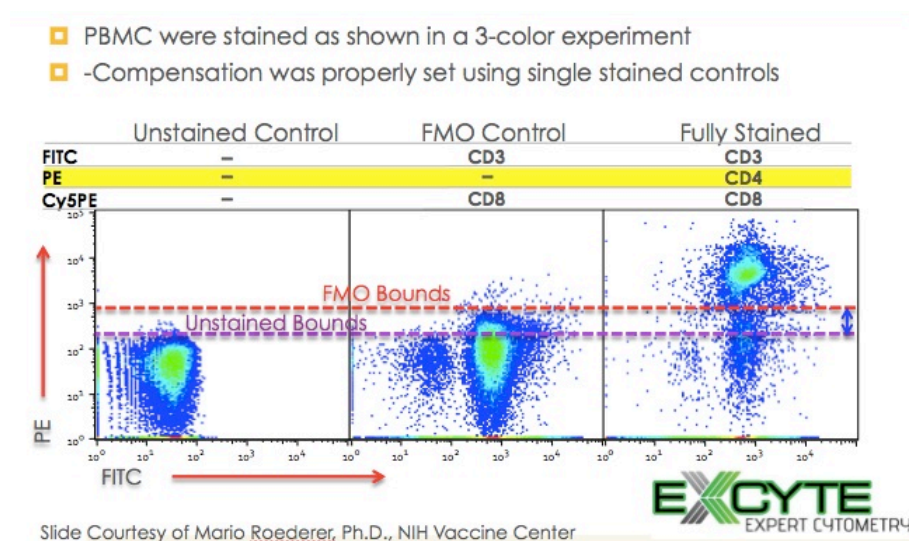


Figure 2.1 Using FMO and isotype for effective gating strategy.

FMO control contains all the fluorochromes in a panel, except for the one that is being measured. For example, in a four-colour panel in Table 2.2., there would be four separate FMO controls:

Table 2.2 Experimental design with FMO for late passage cells

Antigen	APC	FITC	PE-VIO	PE
CD44 FMO	-	CD24	CD133	CD166
CD24 FMO	CD44	-	CD133	CD166
CD133 FMO	CD44	CD24	-	CD166
CD166 FMO	CD44	CD24	CD133	-

2.2.3 Cell Viability Assay

Cell growth was determined by PrestoBlue® Cell Viability Reagent (Thermofisher). This cell permeable resazurin-based solution (PrestoBlue®) indicates the cell viability via quantitative measurement of the proliferation of cells by assessing the reducing power of viable cells. When added to cells, the PrestoBlue® reagent is modified by reducing environment of the viable cell and turns red, becoming highly fluorescent. Cancer cells SNU-1, NCI-N87 (both in ATCC RPMI-1640 Medium) and KATO III (in ATCC Iscove's Modified Dulbecco's Medium) were

plated into 24-well plates at cell number: 2.5×10^4 , 5×10^4 , 1×10^5 , 1.5×10^5 , 2×10^5 , and 2.5×10^5 . Cells were incubated for 24, 48 and 72 hours. 1 ml of PrestoBlue was added to each well and the cells were further incubated for 30 minutes respectively for each time point per manufacturer's recommendation. The fluorescence was recorded on a microplate reader (Synergy Hybrid, Biotek) at a wavelength of 535 nm (excitation) and 615 nm (emission). Top read and bottom read format were recorded due to the different adherence nature of the cells (NCI-N87 – adherent, KATO-III – mixed adherent/suspension, and SNU-1 – suspension cells).

2.2.4 Gene Expression Studies

2.2.4.1 RNA extraction

RNA was extracted from at least 1×10^6 cells using the RNAeasy kit (Qiagen). The kit is supplied with Qiagen's proprietary RLT lysis buffer, RW1 RNA precipitation buffer, and RPE RNA washing buffer. Harvested cells were centrifuged at $1500 \times g$ for 5 minutes to pellet the cell. RLT buffer plus (a lysing buffer; added 10 μ l β -Mercaptoethanol (B-ME) to 1ml RLT buffer) was added to the pelleted cells (350 μ l for $< 5 \times 10^6$ cells / 600 μ l for $< 1 \times 10^7$ cells), and vortexed for 30 seconds. The lysate was centrifuged for 3 minutes at maximum speed ($18000 \times g$). The supernatant was removed by careful pipetting, and the lysate was transferred to the genomic DNA Eliminator spin column that came with the RNAeasy kit and placed in a 2 ml collection tube to remove DNA contaminations. Columns were centrifuged for 30 seconds at $8000 \times g$. Columns were discarded, and flow through supernatant (contained RNA) from the centrifugation was kept aside. RNA precipitation was done by adding 1 volume of 70% ethanol (same volume as RLT volume amount used, e.g. 350 μ l/600 μ l). Next, up to 700 μ l of samples transferred to RNAeasy spin column were placed in a 2 ml collection tube, and centrifuged for 15 seconds at $8000 \times g$, and flow through was discarded (the supernatant from the centrifugation does not contain RNA, instead RNA is retained in the RNAeasy column). RNA in the RNAeasy spin column was washed with 700 μ l of RW1 buffer, followed by washing with 500 μ l of RPE buffer; both times centrifuged at $8000 \times g$ for 15 seconds. Precipitated RNA was washed for the final time with 500 μ l RPE buffer, centrifuged for 2 minutes at $8000 \times g$. For RNA collection, RNAeasy spin columns were placed in a new 1.5 ml collection tube, and 30 μ l of RNAase-free water was added to elute the RNA into collection tubes. Samples were centrifuged for 1 minute at $8000 \times g$. Optionally, the column may be spun again with another volume of water, or the RNA elute to maximise RNA collection. Finally, RNA extracts in each tube were labelled

accordingly and quantified by UV spectroscopy (UV-VIS Nano-spectrophotometer, Implen, Essex, UK) with purity assessed from 260/280nm and 260/230nm ratios. Extracts were stored at -80°C until used.

2.2.4.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

cDNA strands were synthesized by using a reverse transcriptase kit, Superscript II enzyme (ThermoFisher). RNA extracts (100-500 ng) from each sample were added to 300 ng of random primers, heated for 5 min at 65°C and then quickly placed on ice. Each tube (containing the RNA extracts and random primers) was incubated with 0.5 mM dNTPs, 1x first strand buffer, 8µM dithiothreitol and 100 units of Superscript II reverse transcriptase (Invitrogen, Life technologies) for 10 min at 25°C, 90 min at 42°C and 15 min at 70°C in a thermocycler (Peltier Thermal Cycler PTC-200, MJ Research, Waltham, Massachusetts, USA). An mRNA reverse transcription kit was used for RT reaction according to the manufacturer's protocol (Taqman, Applied Biosystems, Life Technologies). Quantitative-Polymerase Chain Reaction (qPCR) was performed using Taqman probe pre-designed gene expression assays (as detailed in Appendix A) and FAST PCR master mix (TaqMan, Applied Biosystems, Life Technologies), and measured in a StepOnePlus fast real-time PCR system (Applied Biosystems, Life Technologies) according to the manufacturer's protocol. GAPDH was used to normalise cellular mRNA expression respectively (Appendix A). Gene expression was quantified using the delta-CT method.

2.2.5 Statistical Analysis

Data were obtained from measurements made in at least four biological replicates and presented as a mean value, $\bar{x} \pm$ Standard deviation (SD). Raw data were recorded and organized in Excel 2016 Microsoft Office program. The statistical analysis was conducted in GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA). The normal distribution of the data sets was checked using the Shapiro-Wilk normality test. Then significant differences ($p < 0.05$) were determined using one-way analysis of variance (*ANOVA*) followed by a Dunnett post-hoc test, or a linear trend analysis and two-way *ANOVA* followed by a Bonferroni post-hoc test. Pearson's correlation coefficient test was used for the correlation analysis.

2.3 Results

2.3.1 Cell Line Characteristics

Essential characteristics of these cells (SNU-1, KATO-III, NCI-N87) have already been reported by their original authors [22], [39], [211]. However, as each culture condition can be different and may affect the cells characteristics, I began this work by identifying the morphology of the cells used in this study. From my observation, SNU-1 has rounded cells grown in suspension, NCI-N87 is an adherent type of cells, grown as a tightly knit epithelial adherent monolayer, whereas KATO-III is a mixture of adherent and floating spherically-shaped suspension cells. Doubling time for SNU-1, KATO-III, and NCI-N87 is 24 hours, determined using various seeding density of 2×10^5 , 1.5×10^5 and 1×10^5 cells (See figure 2.2a, b). The linear regression in Figure 2.2a shows that the increase in cell number is directly proportional to the fluorescence intensity value.

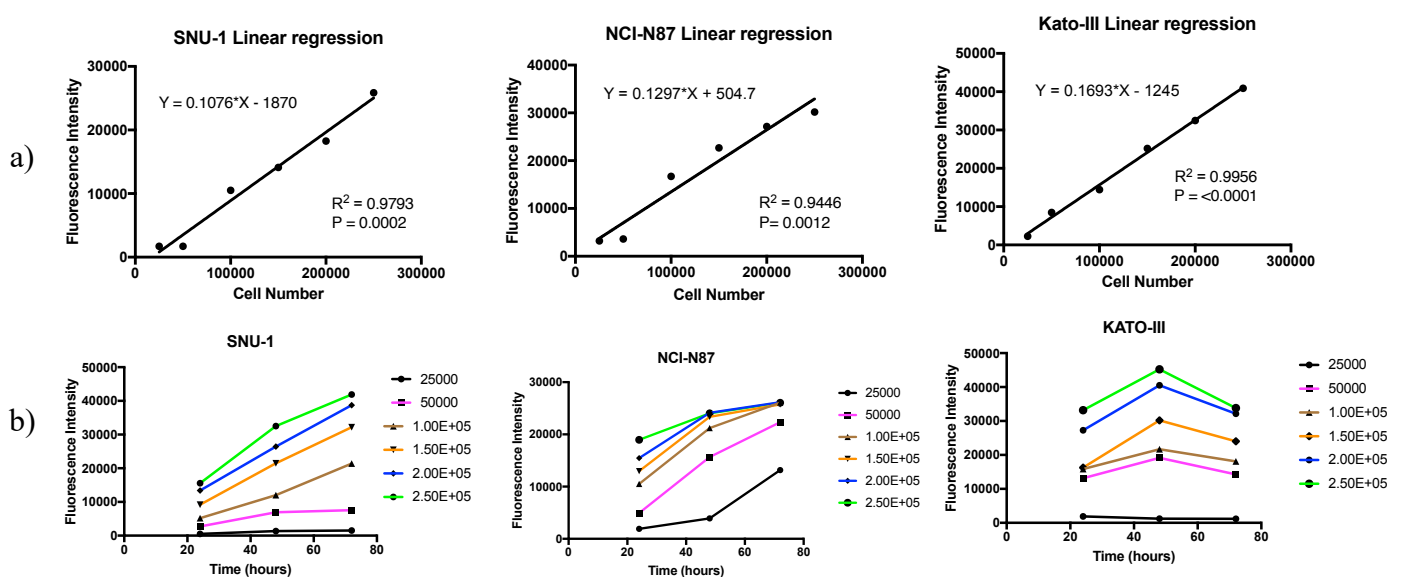


Figure 2.2 a) Linear regression of SNU-1, KATO-III, and NCI-N87 at different seeding densities. b) SNU-1 Growth at Different Seeding Densities and Time Points. Values are $n = 1$.

KATO-III cell proliferation declined after 48 hours, indicated by the loss of fluorescence intensity (Figure 2.2.a). I suspect this is due to the two existing types of cell population competing for nutrients in the culture. Therefore, it was essential to investigate the two existing cell types individually.

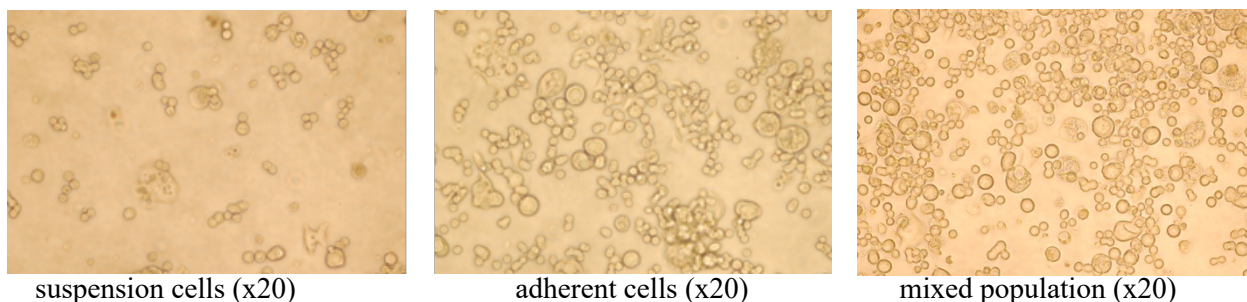


Figure 2.3 KATO-III cell types: suspension cells, adherent cells, and mixed population.

Firstly, I observed the floating suspension cells and noticed they were shaped spherically, with only a few cells appearing to be grouped, while the majority being dispersed. However, the adherent cells were strictly together, almost forming small colonies (Figure 2.3). Therefore, I separated the suspension and adherent KATO-III populations by pipetting out the medium, which contains the suspension cells, while I trypsinised the adherent cells in the T75 cell culture flask and cultured them individually. Both individual cultures for adherent and suspension cells reverted to their normal proportions as mixed populations (with both cell types) within 72 hours. Figure 2.4 shows that the two cell types proliferate differently, with the suspension cells proliferating more rapidly than the adherent cells within a 72-hour culture.

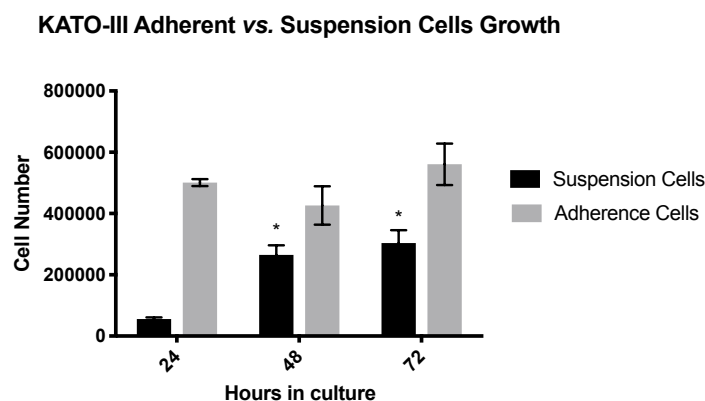


Figure 2.4 Suspension and adherent cells growth at different time points. Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

2.3.2 Cell Surface Marker Expression

Based on the published reports of possible candidate cell surface markers for CSCs [41], [95], [109], [112], [133], [212]-[214], I decided to determine the expression of the CD24, CD44, CD166, and CD133 markers in SNU-1, NCI-N87, and KATO-III cell lines,. The gating strategy is explained in Figure 2.5. The results for these markers are shown in Table 2.3. KATO-III adherent cells at early passage showed the highest expression of CD44 with up to

94.7% of cells expressing CD44, while the suspension cells showed as little as 3.1%. SNU-1 expressed the highest expression of CD24 and CD166 at 8.2% and 10.5% respectively. However, these surface marker expressions diminished at the late passage (P44). NCI-N87 showed 10.1% CD166 expression at early passage and slightly reduced expression at later passage. Thus, from Table 2.3, it is known that the expressions of markers of interest are reduced at late passage for all the cell lines. Therefore, to identify optimal amount of positive populations, cells from early passages are recommended for future testing.

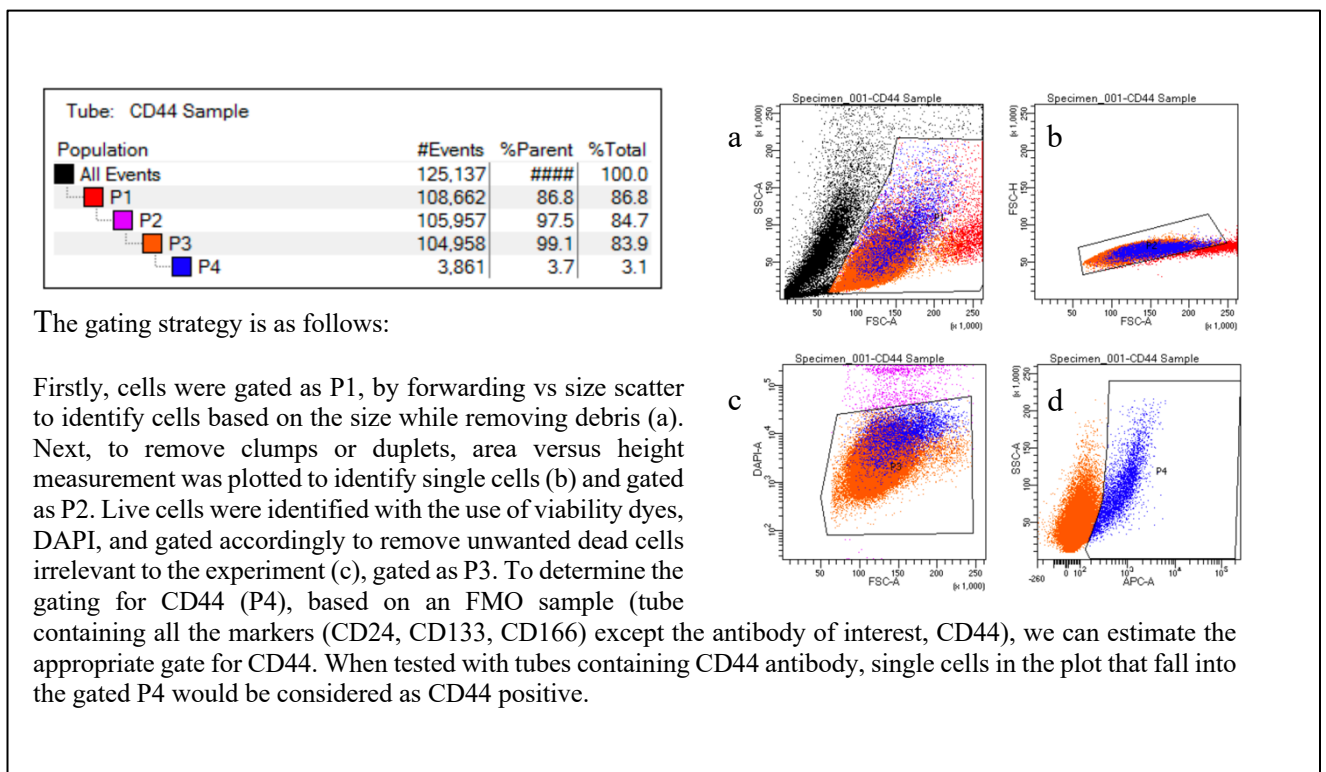


Figure 2.5 Gating Strategy for CD markers, for example, CD44.

Table 2.3 Surface marker expression of CD24, CD44, CD133, and CD166 on gastric cancer cell lines. The percentages (%) represent the positive population of corresponding CD markers (CD24, CD44, CD133, and CD166) in unsorted cell lines. Values are calculated from the average of three readings ($n = 3$). (2-way Repeated Measures ANOVA with Passage (P10 and P44) and CD Marker (CD24, CD44, CD133, and CD166) as independent variables was performed to determine whether the percentage of positive population changes with time. Details of this analysis can be found in the appendix.

Early Passage (P10)				
	CD24%	CD44%	CD133%	CD166%
SNU-1	8.2	3.1	0.1	10.5
KATO-III (adherent)	0.2	94.7	1.3	9.1
KATO-III (suspension)	0.3	3.1	0.03	2.9
NCI-N87	3	0.4	1.4	10.1
Late Passage (P44)				
	CD24%	CD44%	CD133%	CD166%
SNU-1	0.3	0.1	1.1	0.1
KATO-III (adherent)	0.8	2.3	0.6	0.3
KATO-III (suspension)	0.2	0.1	0.02	1.8
NCI-N87	1.8	0.1	0.1	7.7

2.3.3 Expression of stemness gene

The flow cytometry analysis revealed that SNU-1 and KATO-III have the highest expression of cell surface marker related to CSCs, namely CD24, CD44, and CD166. Therefore, I hypothesized that the highest expression of the stemness genes might be observed in these two cell lines. To investigate the stemness gene expression in these GC lines, I screened for *SOX2*, *OCT3/4*, and *NANOG* expression. These genes are known to be critical regulators for stem cells and CSCs [68] in gastric cancer.

Figure 2.6 shows that, in SNU-1 cells, each gene was expressed significantly differently over 72 hours of culture ($P < 0.001$). Over 72 hours of culture, *OCT3/4* expression was the highest at 72 hours ($P < 0.05$). *SOX2* expression was also the highest at 72 hours ($P < 0.001$) but the pattern of change was different: *SOX2*'s increase over time was gradual, while *OCT3/4* remained relatively stable between 24h and 48h time point and spiked rapidly at 72h time point. In contrast, *NANOG* expression was maintained over the 72 hours of culture.

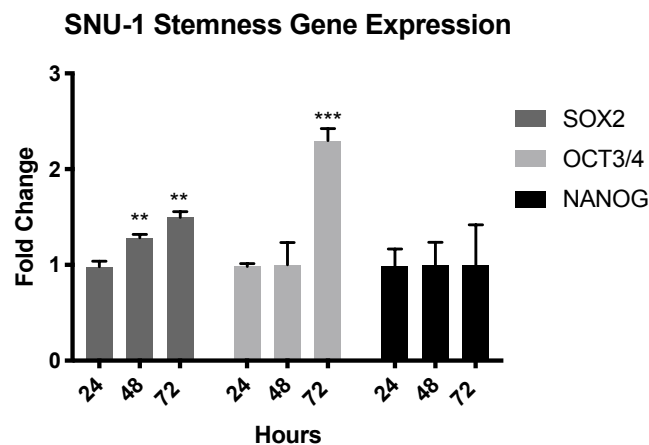


Figure 2.6: Fold change expression of stemness marker *SOX2*, *OCT3/4* and *NANOG* in SNU-1 over 72 hours vs. 24 hours. Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). 2-way Repeated Measures ANOVA with Time (24, 48, 72 hours) and Gene (*SOX2*, *OCT3/4*, and *NANOG*) as independent variables was performed to determine whether gene expression changes with time.

KATO-III Different Cell Types Stemness Expression

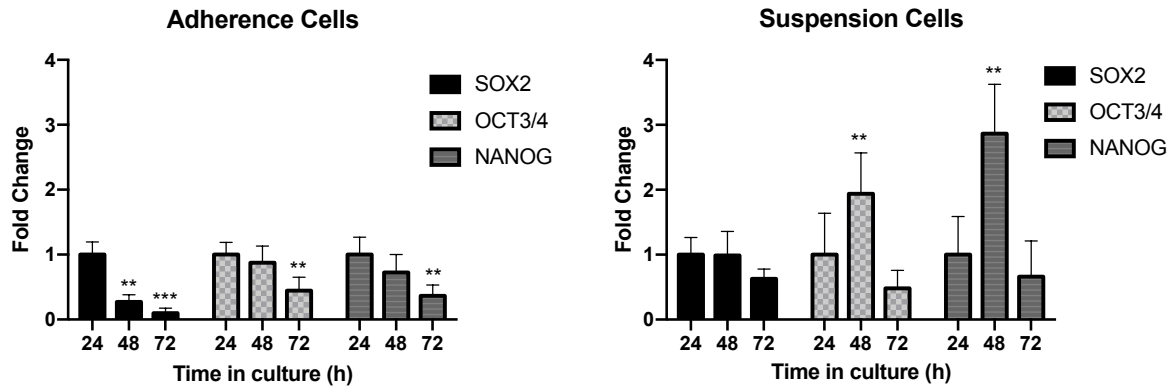


Figure 2.7 Relative mRNA level expression of SOX2, OCT3/4 and NANOG in KATO-III adherent and suspension cells over 72 hours. Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). 2-way Repeated Measures ANOVA, performed separately for adherence and suspension cells, with Time (24, 48, 72 hours) and Gene (SOX2, OCT3/4, and NANOG) as independent variables was used to determine whether gene expression changes with time.

I also examined whether there was a difference between stemness factor expression in adherent and suspension cells in KATO-III (Figure 2.7). In suspension cells, *OCT3/4* and *NANOG* showed increased fold change expression at 48 hours in comparison to 24 hours but the expression of both genes decreased below the 24-hour level at 72 hours. On the other hand, in adherent cells, expression of all three stemness genes gradually decreased over 72 hours period.

2.4 Discussion

2.4.1 Cell proliferation

Cell proliferation and differentiation are found to be reliant on the cell seeding density [24], [215], [216]. By having a higher density of initial seeding, as observed in Figure 2.2, increased cell-cell contact can positively regulate proliferation [217]. It was shown that by increasing the seeding density for all the cell lines, from 2.5×10^4 to 2.5×10^5 cells per well, each cell line exhibits positive growth for the first 24 hours. However, too high a seeding density suppresses proliferation.

2.4.2 Side population in gastric cancer cell lines.

Each of the cell lines examined has different expression patterns of CD24, CD44, CD133, and CD166. Although according to some studies these markers may not necessarily define a cancer stem cell population [105], [209], the other studies show that this subpopulation could be a prospective subject of stem cell-like population study within a cancer cell line [41], [99]. For example, it has been reported that when transplanted into the skin and stomach of SCID mice, a side population of CD44⁺ from NCI-N87 was found to generate tumours in both the skin and stomach after 8-12 weeks, while the CD44⁻ cells did not [41]. In another report, CD24⁺ cells were found to exhibit enhanced chemotherapy-resistance, self-renewal and tumorigenic capacity typically regarded as CSC hallmarks ([1], [153], [218]), both *in vitro* and *in vivo*, compared to CD24⁻ subpopulations, which did not display such properties [112]. Additionally, it has been reported that side populations isolated in KATO III have clonogenicity and self-renewal ability, similar to stem cell-like nature [23]. Therefore, the identification of the CSC markers of CD24, CD44, CD133, CD166 in SNU-1, KATO-III, NCI-N87 (Table 2.3) provides a direction for me to investigate further the stemness population within the cell lines. Notably, as shown in Table 2.3, the cell surface marker phenotype in these gastric cell lines can also change over time, with progressing from early towards later passage. This was an interesting observation in this study as it suggests that surface protein marker can change. This finding is similar to the results of another study, where some ovarian cancers with only CD133⁺ tumorigenic population could later produce CD133⁻ non-tumorigenic cells with the passage [219]. If cell phenotype commonly changes with a passage, this may explain the possible inconsistencies among studies investigating sub-populations within cancer cells.

Notably, a high percentage of cells expressing CD44 is not surprising, as several gastric cell lines such as MKN-45 and MKN-74 can have a high level of CD44 population too, as high as 94%, whereas NCI-N87 can show a percentage as low as 5% of the CD44⁺ population [41]. This is possibly due to the nature of CD44 cell surface glycoprotein, which is involved in cell-adhesion [220]. Alarming, this suggest that the so-called CSC cells can be as abundance as non-CSC cells. This is problematic from CSCs model standpoint, where it suggests that rare and small population within cancer have the capacity to drive tumour progression [90], [221]

In this study, the highest CD24 and CD166 expression was found in the early passage SNU-1 cell line but decreased at later passage (Table 2.3). Since SNU-1 was sourced from primary gastric carcinoma, there is a possibility that these markers expressed at early passage SNU-1 play an essential role in the subpopulation's migration and invasion capabilities. CD24 regulates CXCR4, an essential receptor in stem cell homing and tumour metastasis [55], and influences the cell's migrating and invasion abilities [95], [108], [222] – taken together constitute an essential characteristics of primary sourced culture [160].

2.4.3 Stemness Gene Expression

Various studies have established the association between stemness transcription such as *SOX2*, *OCT3/4*, and *NANOG* in gastric carcinoma [64], [68], [128], [129]. Notably, the expression of transcription factors such as *SOX2*, and *OCT3/4* in both SNU-1 and KATO-III (Table 2.6 and Table 2.7) could suggest these transcription factors' involvement in both cell lines' maintenance. These transcription factors have been previously known to be essential in pluripotent cell development and maintenance, in addition to their self-regulating roles in determining other cell types [88], [223]. However, the expression of pluripotent genes detected in this study is likely a product of the essential drivers underlying self-renewability and production of differentiated progeny in differentiated cells [224]. Recent studies found that adult stem cells may exhibit broader potential or plasticity (similar to the one observed in embryonic stem cells) than we initially thought [152]. These findings pose a question whether, at transcriptional level, all stem-like cells are actually very similar. In suspension type cells of KATO-III, higher fold change of *OCT3/4* and *NANOG* mRNA was detected than in the adherent type cells. A possible explanation to this is that in the side population (SP) of KATO III, the expression of *OCT3/4* in side population (SP) cells was higher than in non-SP cells,

suggesting that SP cells had some characteristics of undifferentiated cells [23]. Therefore, to study the expression of these stemness markers and the subpopulations is an interesting direction for further investigation, in which the cell line of interest must be sorted and have the corresponding subpopulation's stemness markers examined.

2.5 Conclusion

The work in this chapter confirms the previous characterization of these cell lines and presents additional findings to understand the stemness factors involved. The fact that the morphological difference in KATO-III cell types could also indicate differences in surface marker and stemness gene expression is a novel finding of this study. Apart from the established knowledge on the importance of passage number that can affect cells' phenotype [21], and the regulation of the expression of cell surface markers, [225] little is known of the expression of stemness markers in the subpopulations found in GC cell lines. By understanding the potential connection between stemness expression and subpopulations, we could elucidate whether the mechanistic process of phenotypic change (when subject to induction) may affect these sub-populations. Moving forward, I chose SNU-1 as the best cell line candidate for future experiments due to the expression of two main CSC surface markers, CD24 and CD166. In contrast to KATO-III, which could unquestionably be a more interesting subject for investigation, the stemness population of CD44 population has been widely characterised and studied in previous studies. In addition, it is unclear to me if the adherent cells may transform into suspension cells or *vice versa*. If such phenomenon indeed occurs, further investigation is needed to determine at which passage these morphological changes take place. Therefore, I chose SNU-1 cells as they may prove more reliable and predictable in future studies. Furthermore, SNU-1 as diffuse-type GC, with poorly differentiated cells has been associated with an increased stemness capacity. Thus, I will investigate the side populations of SNU-1 by critically analyse which population expresses the highest stemness factor. Therefore, before exploring the plasticity aspects of the SNU-1 cancer cells further, analysis of subpopulations will help to characterize the internal factors that may influence the phenotypic change of the gastric cancer cell line. In the next chapter, I will attempt to investigate these subjects.

3 Characterization of SNU-1 Subpopulations

3.1. Introduction

Cancer stem cells (CSC) are proposed to exist as a small subgroup of cell population with the characteristics of stem cells, which have unlimited self-renewal and replicative potential that could trigger tumour growth, metastasis and tumour recurrence [226]. As such, in 2006 American Association for Cancer Research (AACR) summarised the CSC topic: "In the cancer stem cell model of tumours, there is a small subset of cancer cells, the cancer stem cells, which constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and maintain the tumour" [227]. In 1997, Bonnet and Dick presented the first conclusive evidence of enriched leukaemia stem cell subpopulations expressing surface marker $CD34^+CD38^-$ (expressing CD34 but not CD38) within the leukaemia cells, capable of forming tumours after implantation *in vivo* in NOD/SCID mice [90]. This surface marker is similar in phenotype to normal SCID-repopulating cells. Thus, they showed that the leukemic transformation was driven by normal primitive cells with stem cells' capacity to self-renew and form tumour, rather than by committed progenitor stem cells. Later, similar findings of CSCs were reported in a solid tumour in breast cancer [91] and brain [228]. These earlier studies showed that it is possible to isolate distinct subpopulations that differ in their cell-surface protein antigen profiles from a single tumour using cell surface marker. However, CSC phenotype representation may vary considerably between cancer subtypes. For example, within breast cancer subtypes, the prevalence of cells with various CD44/CD24 phenotypes ($CD44^+/CD24^-$, $CD44^-/CD24^+$ and $CD44^+/CD24^+$) accounts for 13% of the breast cancer subtypes; in addition, 31% of the tumours have $CD44^+/CD24^-$ cells with various percentages (from 0 to almost 100%) of tumour cells [229]. Similarly, I found that CD44 subpopulation accounts for approximately 94% of adherent KATO-III cells (see Chapter 2, Table 2.3). Thus, in some tumours, CSCs could be as abundant as non-CSCs. Several reports suggested a similar idea. For example, in the study of isolated lymphoma cell transgenic mice, in which the cells were injected into the host animal, regardless of the number of cells injected (from 10 to 10⁵ cells), a tumour was formed in all recipients [230]. Similarly, in another study, as many as 25% of the melanoma cells isolated from primary and metastatic melanomas, formed tumours and had CSCs properties [221]. Thus, it was suggested that tumour growth need not be determined only by rare cancer cells. In the case of transplanted animals, the issue is often underestimated, especially when transplantation is performed in severely immunocompromised mice, hence

questioning the relevance of rare CSCs. These findings are in contrast with the CSC model, which suggested that tumorigenic cells can be found in a small subpopulation and that these cells have tumorigenic potential enabling them to form a tumour in xenotransplantation in NOD/SCID mice [96], [114], [221].

Secondly, tumour malignancy, in general, is measured by the cancer cells' degree of dedifferentiation in a tumour [231]. Therefore, regulators of differentiation are active contributors in the CSC biology. Some evidence suggests that cells arising from mammary epithelial cells possess both stem and tumorigenic characteristics of CSCs [232], [233], which may indicate that CSCs are preferentially associated with the state of differentiation such as epithelial-mesenchymal-transition (EMT) process.

To connect the stochastic and CSC model, some scientists have proposed that the transitional state between CSC and non-CSC is better suited for a phenotypic plasticity model [142], [148], [234], which can potentially explain the state of heterogeneity and (de)differentiation that occurs within some tumours. Following this paradigm, Ralph and colleagues demonstrated the potential of phenotypic plasticity by showing that in order for prostate cancer cells to escape destruction, the cells may undergo developmental reprogramming under therapeutic challenge to alter themselves to a more stem-like state [235]. Therefore, it remains a question whether CSC-like subpopulations in SNU-1 (CD24 and CD166) can be defined by the stemness expression of the subpopulations? To what degree would the expression of stemness genes (*SOX2*, *OCT3/4*, and *NANOG*, *ABCG2*, *ALDH1*, *LGR5*), xenobiotic response and drug metabolism genes (*CYP1A1*, *CYP1B1*, *AhR*, *AhRR*, *ARNT*), along with EMT-inducer genes (*Snail*, *CTNBN1*, *MYC*, *SHH*) experience loss during cell culture? These questions are critical to providing a better understanding on these subpopulations within the gastric cancer cell line, SNU-1, [if some or all of its population had the necessary expression of related genes to support the phenotypic plasticity idea of reversible phenotype.

3.1.1. Hypotheses

1. A sorted and enriched populations of CD24 and CD166 will express high levels of stemness genes.
2. Expressions of essential genes involved in xenobiotic response and EMT in each sorted population will vary, and their expressions will change upon culture.

3.2. Materials and Methods

3.2.1. Sample preparation for magnetic sorting of SNU-1 subpopulations

CD166 microbead, purchased from Miltenyi Biotec, contains 1 ml CD166-biotin, human (130-106-574, monoclonal CD24) antibody conjugated to biotin (clone: REA442; isotype: recombinant human IgG1), and 2 ml of Anti-Biotin Microbeads (130-090-485, MicroBeads conjugated to monoclonal anti-biotin antibodies; isotype: mouse IgG1). CD24 microbead kit (130-095-951) was also purchased from Miltenyi Biotec. The kit comprised of 1 ml CD24-Biotin, human (monoclonal CD24) antibody conjugated to biotin (clone: 32D12; isotype: mouse IgG1), and 2 ml Anti-Biotin MicroBeads (MicroBeads conjugated to monoclonal anti-biotin antibodies; isotype: mouse IgG1). A buffer solution containing phosphate-buffered saline (PBS), pH7.2, was prepared with the addition of 0.5% bovine serum albumin (BSA) for use in the sorting process. This solution was used throughout the magnetic separation process. LS columns (130-041-306, Miltenyi Biotec) were used for positive selection of cells strongly expressing the CD24 antigen. Cell lysate obtained from cell culture was washed once with PBS and centrifuged at $300\times g$ for 10 minutes. The cell pellet was resuspended in the sorting buffer made earlier, and cells were passed through moistened 30 μm nylon mesh (Pre-Separation Filters (30 μm), 130-041-407, Miltenyi Biotec) to remove any cell clumps which could clog the column during the sorting process. Once cell number had been determined, minimum 1×10^7 cells were used for the magnetic sorting. The suspension cells were centrifuged at $300\times g$ for 10 minutes, and the supernatant was aspirated completely. The cell pellet was resuspended in 40 μL of the buffer, and 10 μL of CD24-Biotin. CD166-biotin was added (for 10^7 total cells). Staining antibodies (CD24-FITC, CD166-PE, refer to Chapter 2 for details) was added as well to the mix for flow cytometry analysis at the end of magnetic separation. The solution was mixed well and incubated for 15 minutes in the dark in the refrigerator at 2–8 °C. Higher temperature or more prolonged incubation may promote non-specific cell labelling. Later, the cells were washed with 2 ml of the sorting buffer and centrifuged for $300\times g$ for 10 minutes, and the supernatant was removed entirely. The cells were then resuspended in 500 μL of the buffer, and magnetic separation process begins.

3.2.1.1. Magnetic separation of subpopulations CD24 and CD166

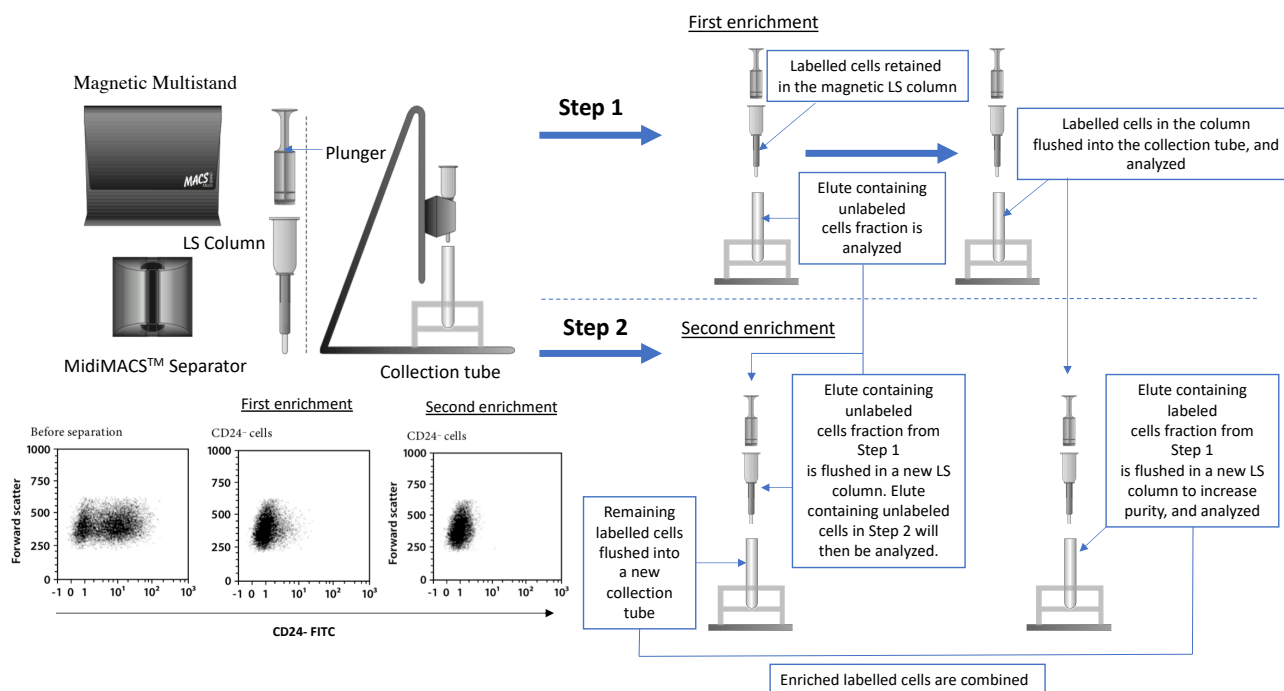


Figure 3.1: Magnetic separation process to isolate and enrich CD24 and CD166 subpopulations.

LS column was placed in the magnetic field of the MACS separator. The column was activated by being rinsed with 3 ml of the sorting buffer made earlier. Slowly, the cell suspension was added onto the column, and flow through was collected. The flow-through will contain unlabelled cells. To maximise the efficiency collection of unlabelled cells, the column was washed with 3 ml sorting buffer three times. The LS column was removed from the separator and placed on a new collection. 5 ml buffer was pipetted onto the LS column, and immediately, cells were flushed out from the LS column by using the plunger supplied with the column. This step fulfilled the first enrichment process. The labelled fraction of cells in the collection tube was analysed for CD24⁺/CD166⁺ percentage with a flow cytometer, Moxi Go II (Orflo, Moxi Go OS v4.3). For the second enrichment process, and to increase the purity of the labelled fraction of cells, the eluted labelled cell fraction was passed over the second LS column, and separation procedures described above were repeated. The eluted unlabelled cell fraction was also passed over to another second LS column, following the same separation procedure. Once the elution process finished, the LS columns was placed onto a new collection tube and the remaining labelled cells in the magnetic column were flushed out.

3.2.2. Flow cytometry analysis

Labelled fraction and unlabelled fraction of cells from magnetic separation were processed for flow cytometry analysis. Firstly, cell counts were performed using an automated cell counter machine (Moxi Z from ORFLO Technologies), and 1×10^6 cells were added into each FACS tube and incubated on ice for 15 minutes. New medium (1 ml) was added to as many tubes as required and centrifuged for $500 \times g$ for 5 minutes. PBA (30 μ l of 1% Bovine serum albumin dissolved in Phosphate buffered solution) containing 3 μ l of FcR Blocking reagents (Miltenyi Biotec) was added to each tube containing the cells to reduce non-specific binding of the antibodies. PBA also stabilizes the cell membrane and prevents antibodies from sinking into the membrane.

Next, supernatants were discarded, and 10 μ l of an antibody of each CD markers (refer to Table 3.1 for concentrations) diluted in 100 μ l of staining buffer were added to 1×10^6 cells' pellet in each respective tube labelled with the corresponding CD marker being used. Then the tubes were incubated for 30 minutes on ice. It is worth noting that antibodies were titrated to determine optimal antibody concentration for live-cell labelling to achieve the best discrimination between positive and negative cells, while keeping nonspecific antibody binding to a minimum, according to the protocol's recommendation [210]. Finally, 500 μ l PBA was added to each tube, and the samples were centrifuged for $400 \times g$ for 15 minutes, and the supernatant was discarded to remove excess antibodies. Cell pellets were resuspended in 1 ml of PBA and were examined with flow machine (BD FACS Fortessa). The results were analysed using FlowJo software, version 10.1r1, on Mac OS X El-Capitan Operating System, version 10.11.5.

Table 3.1 CD markers used in the experiments:

CD Markers	Supplier	Supplier items code	Concentration (per 10 μ l)
FITC Mouse Anti-Human CD24	Becton Dickinson	555427	0.2ug/ μ l
PE Mouse Anti-Human CD166	Becton Dickinson	559263	0.0125ug/ μ l

Fluorescence Minus One (FMO) Control) is used in this experiment. The FMO control is employed to correctly interpret flow cytometry data, and especially in the case in which the population of interest is meagre; it acts as a reasonable quality control. It is used to identify and gate cells in the context of data spread due to the multiple fluorochromes in each panel. An FMO control contains all the fluorochromes in a panel, except for the one that is being measured. Therefore, since there were only two antibodies used, the FMO for each sample would be the unstained sample without any antibody.

Table 3.2 Experimental design with FMO for SNU-1 cells.

Antigen	FITC	PE
CD24 FMO	-	CD166
CD166 FMO	CD24	-

3.2.3. Gene Expression Studies

3.2.3.1. RNA extraction

RNA was extracted from at least 1×10^6 cells using the RNAeasy kit (Qiagen). The kit is supplied with Qiagen's proprietary RLT lysis buffer, RW1 RNA precipitation buffer, and RPE RNA washing buffer. Harvested cells were centrifuged at $1500 \times g$ for 5 minutes to pellet the cell. RLT buffer plus (a lysing buffer; added $10 \mu\text{l}$ β -Mercaptoethanol (B-ME) to 1ml RLT buffer) was added to the pelleted cells ($350 \mu\text{l}$ for $< 5 \times 10^6$ cells / $600 \mu\text{l}$ for $< 1 \times 10^7$ cells), and vortexed for 30 seconds. The lysate was centrifuged for 3 minutes at maximum speed ($18000 \times g$). The supernatant was removed by careful pipetting, and the lysate was transferred to the genomic DNA Eliminator spin column that came with the RNAeasy kit and placed in a 2 ml collection tube to remove DNA contaminations. Columns were centrifuged for 30 seconds at $8000 \times g$. Columns were discarded, and flow through supernatant (contained RNA) from the centrifugation was kept aside. RNA precipitation was done by adding 1 volume of 70% ethanol (same volume as RLT volume amount used, *e.g.* $350 \mu\text{l}/600 \mu\text{l}$). Next, up to $700 \mu\text{l}$ of samples transferred to RNAeasy spin column were placed in a 2 ml collection tube, and centrifuged for 15 seconds at $8000 \times g$, and flow through was discarded (the supernatant from the centrifugation does not contain RNA, instead RNA is retained in the RNAeasy column). RNA in the RNAeasy spin column was washed with $700 \mu\text{l}$ of RW1 buffer, followed by washing with $500 \mu\text{l}$ of RPE

buffer; both times centrifuged at 8000×g for 15 seconds. Precipitated RNA was washed for the final time with 500 µl RPE buffer, centrifuged for 2 minutes at 8000×g. For RNA collection, RNAeasy spin columns were placed in a new 1.5 ml collection tube, and 30 µl of RNAase-free water was added to elute the RNA into collection tubes. Samples were centrifuged for 1 minute at 8000×g. Optionally, the column may be spun again with another volume of water, or the RNA elute to maximise RNA collection. Finally, RNA extracts in each tube were labelled accordingly and quantified by UV spectroscopy (UV-VIS Nano-spectrophotometer, Implen, Essex, UK) with purity assessed from 260/280nm and 260/230nm ratios. Extracts were stored at -80°C until used.

3.2.3.2. *Reverse Transcription Polymerase Chain Reaction (RT-PCR)*

cDNA strands were synthesized by using a reverse transcriptase kit, Superscript II enzyme (Thermofisher). RNA extracts (100-500 ng) from each sample were added to 300 ng of random primers, heated for 5 min at 65°C and then quickly placed on ice. Each tube (containing the RNA extracts and random primers) was incubated with 0.5 mM dNTPs, 1x first strand buffer, 8µM dithiothreitol and 100 units of Superscript II reverse transcriptase (Invitrogen, Life technologies) for 10 min at 25°C, 90 min at 42°C and 15 min at 70°C in a thermocycler (Peltier Thermal Cycler PTC-200, MJ Research, Waltham, Massachusetts, USA). An mRNA reverse transcription kit was used for RT reaction according to the manufacturer's protocol (Taqman, Applied Biosystems, Life Technologies). Quantitative-Polymerase Chain Reaction (qPCR) was performed using Taqman probe pre-designed gene expression assays (as detailed in Appendix A) and FAST PCR master mix (TaqMan, Applied Biosystems, Life Technologies), and measured in a StepOnePlus fast real-time PCR system (Applied Biosystems, Life Technologies) according to the manufacturer's protocol. GAPDH was used to normalise cellular mRNA expression respectively (Appendix A). Gene expression was quantified using the delta-CT method.

3.2.4. Statistical Analysis

Data were obtained from measurements made in at least four biological replicates and presented as a mean value, $\bar{x} \pm$ Standard deviation (SD). Raw data were recorded and organized in Excel 2016 Microsoft Office program. The statistical analysis was conducted in GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA). The normal distribution of the data sets was checked using the Shapiro-Wilk normality test. Then significant differences ($p < 0.05$) were determined using one-way analysis of variance (*ANOVA*) followed by a Dunnett post-hoc test, or a linear trend analysis and two-way *ANOVA* followed by a Bonferroni post-hoc test. Pearson's correlation coefficient test was used for the correlation analysis.

3.3. Results

3.3.1. Enrichment of the CD24 and CD166 subpopulation

To sort cells with higher purity, I performed the magnetic cell sorting twice, on the same batch of cells using a new column each time (Figure 3.2). Table 4 summarizes the average increase of each population with second enrichment. T-test showed that second enrichment is sufficient to increase the purity of the sorted cells ($n = 3$, $P < 0.001$). In an efficient setup and through precise application of MACS, flow through has a very low residue of CD24+ and CD166+, as shown in Figure 3.3.

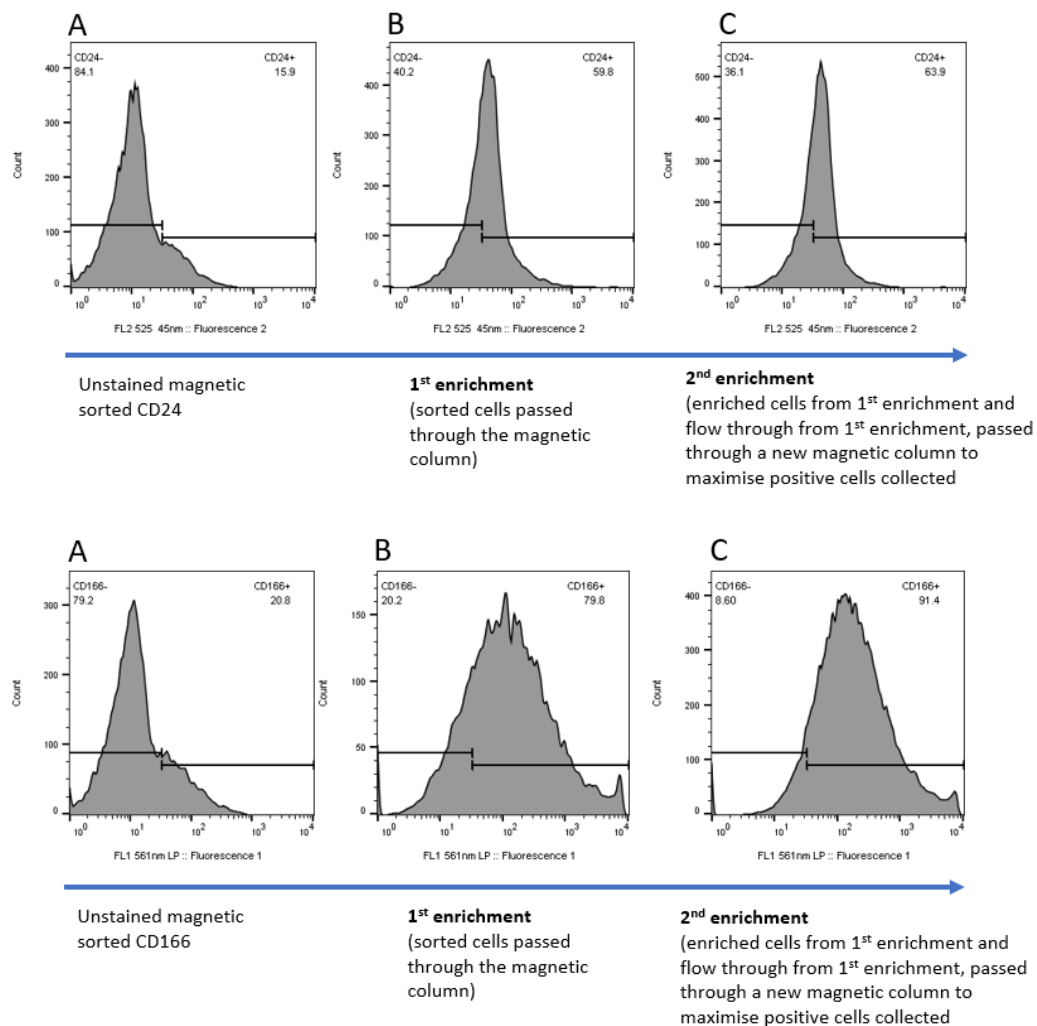


Figure 3.2: Enrichment process of CD24 and CD166 magnetic sorted populations. The positive (+) or negative (-) symbols refer to the populations that express the CD marker (e.g. CD166⁺), or lack the expression of the CD markers of interest (e.g. CD166⁻). The numbers below them (e.g. 91.4, 8.60 etc.) represent the percentage (%) of cells from these total populations.

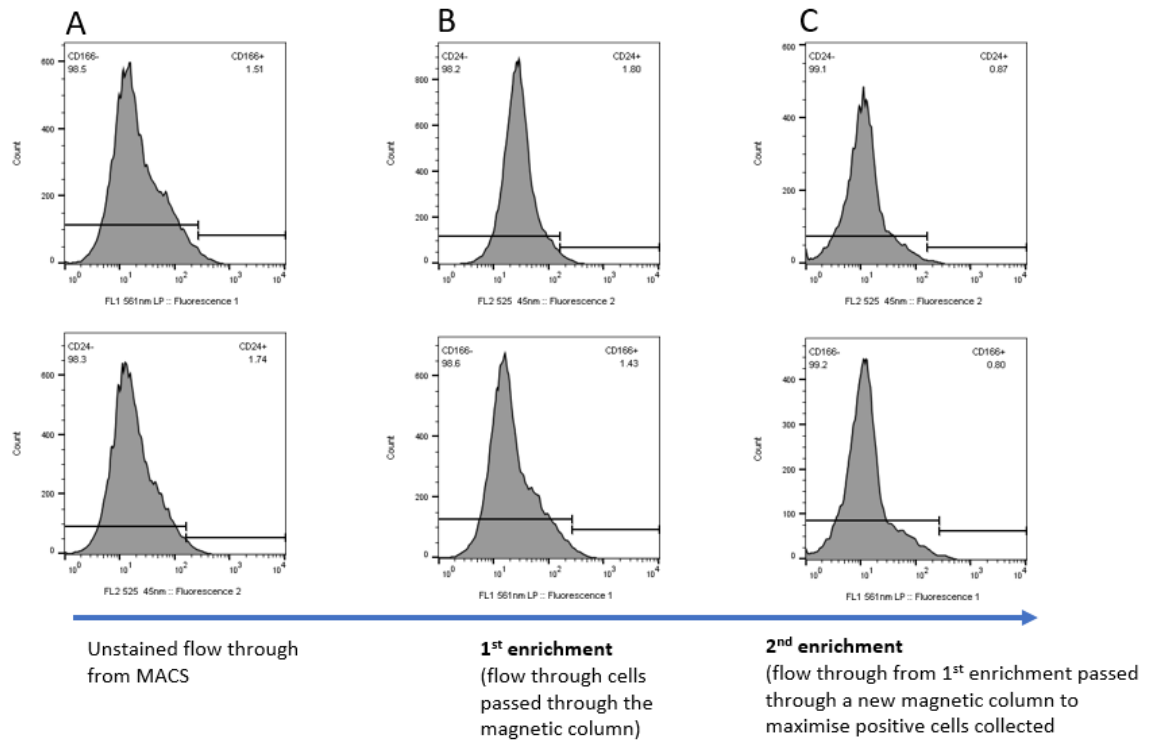


Figure 3.3: Enrichment process of flow through from magnetic sorted cells. The positive (+) or negative (-) symbols refer to the populations that express the CD marker (e.g. CD166⁺), or lack the expression of the CD markers of interest (e.g. CD166⁻). The numbers below them (e.g. 91.4, 8.60 etc.) represent the percentage (%) of cells from these total populations.

Table 3.3: Average percentage of positive population from MACS; $n=3$, $t(5)=11.56$, $P<0.001$

Unstained magnetic sorted	Average 1 st enrichment (%)	Average 2 nd enrichment (%)
CD24 ⁺	50	88.6
CD166 ⁺	61.7	89.3

3.3.2. Can the sorted cells maintain their respective population over time in culture?

To answer this question, sorted cells were cultured up to 72 hours ($n=4$). Cells were stained with corresponding fluorescent antibody and analysed with the flow cytometer. Additionally, mRNA was extracted, and gene expression accessed using QRT-PCR method. Over 72 hours, CD24 and CD166 expression was reduced to ~50%. In addition to that, stemness gene expressions such as *SOX2* and *NANOG* were also reduced (refer to Figure 3.4).

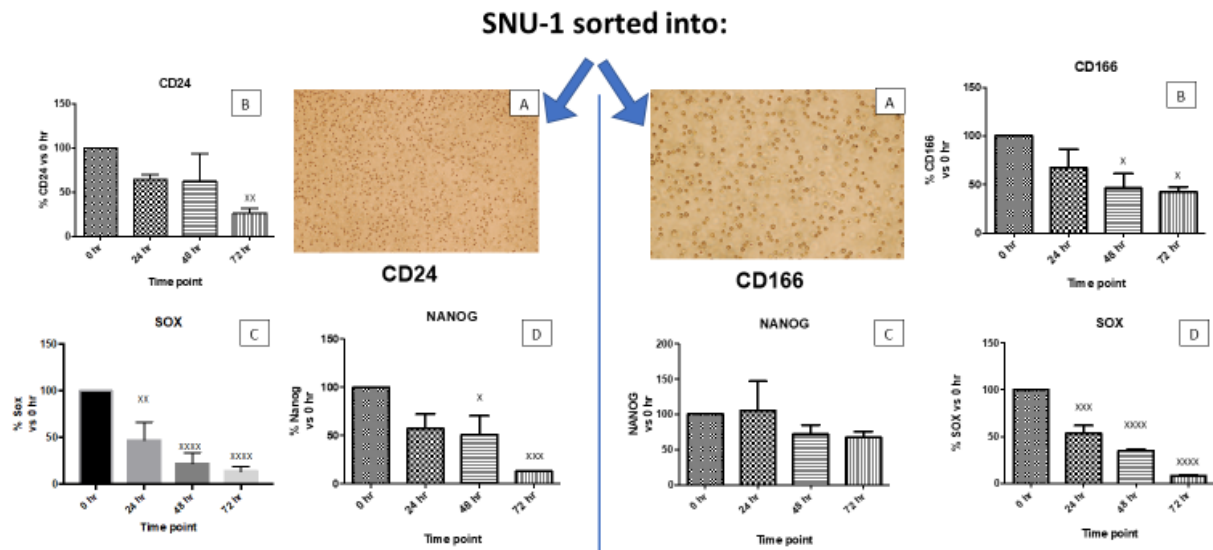


Figure 3.4: (A) Microscopic view (x10) of CD24 & CD166 sub-population sorted. (B) Confirmation of CD24 expression with QRT-PCR over 72 hours. (C&D): SOX2 and NANOG stemness genes expression in the subpopulation over 72 hours. Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). 1×10^9 SNU-1 cells were used for the initial sorting process. One-way Repeated Measures ANOVA was performed to investigate the effect of Time on the expression of the respective genes.

3.3.3. Essential genes expression in the sorted populations of SNU-1

mRNA expressions of genes involved in the regulation of cellular activities were examined in sorted SNU-1 subpopulations. Essential genes include stemness genes (*SOX2*, *OCT3/4*, *NANOG*, *ABCG2*, *ALDH1*, and *LGR5*), xenobiotic response genes (*CYP1A1*, *CYP1B1*, *AhR*, *AhRR*, *ARNT*), and EMT transcription factors (*SNAIL/Snail*, *MYC*, *CTNNB1*, *SHH*). Gene expressions were evaluated on the sorted populations of CD24⁺, CD166⁺, and CD24⁻CD166⁻ (refer to Figure 3.7).

3.3.3.1. Stemness genes

All sorted populations (CD24⁺/CD166⁺/CD24⁻CD166⁻) expressed *SOX2*, *OCT3/4*, and *NANOG* stemness markers. On the contrary, *ABCG2* expression was only found in the CD24⁺ population (Figure 3.7A). *ALDH1* and *LGR5* were hardly detected in all subpopulations even after 40th cycles of QRT-PCR. A gradual decrease in stemness gene expression was found in both CD24⁺ and CD24⁻CD166⁻ sorted populations, whereas no significant change was detected in CD166⁺ subpopulation (Figure 3.7B), except for *NANOG*, which showed significant increase of expression at 48 and 72 hours in comparison to the baseline at 24 hours. Notably, CD24 and

CD166 gene expressions increased in CD24⁻CD166⁻ sorted populations at 48 and 72 hours (Figure 3.7C), and the surface marker expression in CD24⁻CD166⁻ showed that the corresponding markers increased too at 48 and 72 hours (Figure 3.6), indicating the population is steadily recapitulating the former equilibrium of CD24⁺ and CD166⁺ cells. On the other hand, in CD24⁺ and CD166⁺ sorted subpopulation, the expression of the corresponding gene (CD24/CD166) decreased at 72 hours in CD24⁺ and CD166⁺ subpopulation respectively (Figure 3.6A, 3.6B). The expression of CD24 protein surface marker in CD24⁺ population, and the expression of CD166 protein surface marker in CD166⁺ population, which were determined by flow cytometry, showed similar patterns (Figure 3.5).

3.3.3.2. *Xenobiotic response genes*

Expressions of genes such as *AhR* and *CYP1* family (e.g. *CYP1A1*, *CYP1B1*) play a critical role in mediating the toxicity of chemicals from the environment, involved in xenobiotic response and also drug metabolism [191], [236]. These genes are expressed in all sorted populations. In CD24⁺ subpopulation, the expressions of *AhR* and *CYP1* genes decreased over 72 hours in all subpopulations (Figure 3.7D). However, *AhRR* and *ARNT* showed increased expressions in all subpopulations (Figure 3.7D, 3.7E, 3.7F), with notably increased *AhRR* expression in CD166⁺ (Figure 3.7E) and CD24⁻CD166⁻ sorted populations (Figure 3.7F).

3.3.3.3. *EMT-inducer genes*

Despite the lack of *E-Cadherin* expression, all sorted populations expressed *Snail*, *MYC*, and *CTNNB1*, however with varying level of expression. In CD24⁺ subpopulation, *Snail* showed a significant increase in expression only in CD24⁺ subpopulation (Figure 3.7G) but the expression decreased in other subpopulations (Figure 3.7H, 3.7I). On the contrary, *CTNNB1* and *MYC* gene expressions significantly increased only in CD166⁺ subpopulation (Figure 3.7H), indicating that WNT signalling pathway is activated in this subpopulation in comparison to others. Notably, CD24⁻CD166⁻ sorted population showed no increase of any EMT-inducer genes (Figure 3.7I). *SHH* gene was not detected in any subpopulation, which ruled out the involvement of Sonic Hedgehog (SHH) pathway as a potential EMT-inducer.

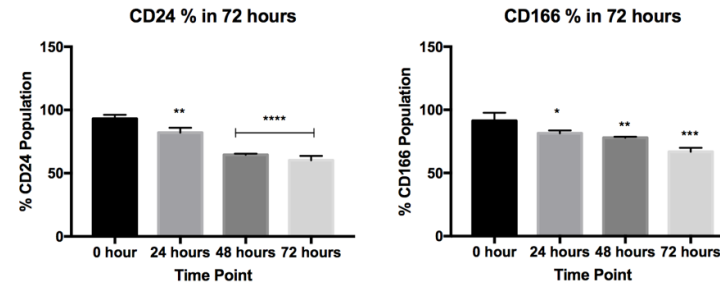
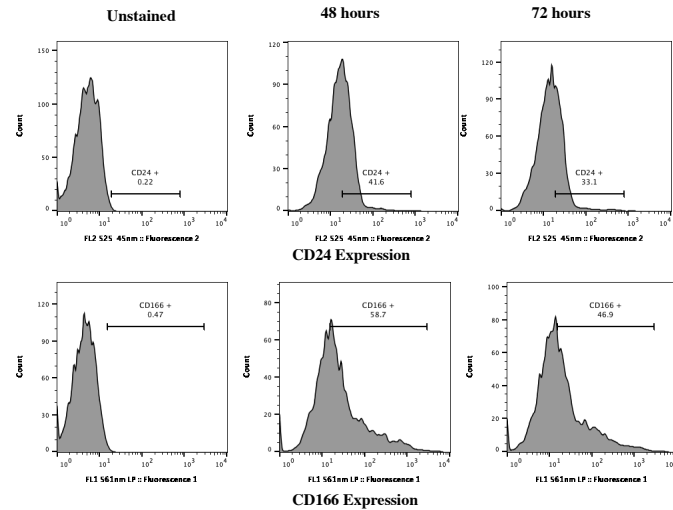


Figure 3.6: Surface protein expression of CD24 and CD166 in CD24⁺ (left) and CD166⁺ (right) subpopulations respectively in 72 hour culture. Values are $\bar{x} \pm SD$, $n = 3$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way Repeated Measures ANOVA was performed to investigate the effect of Time on the percentage of positive population of the respective markers.

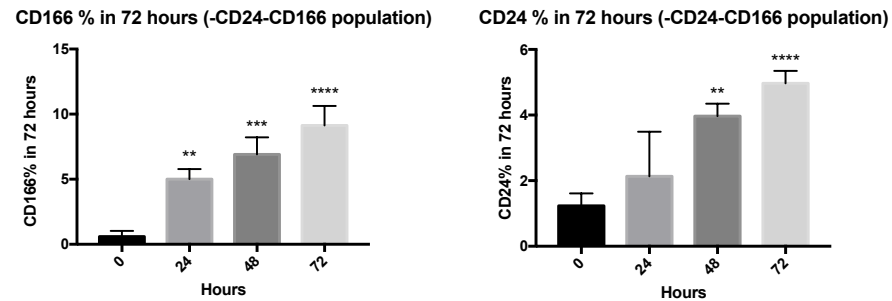
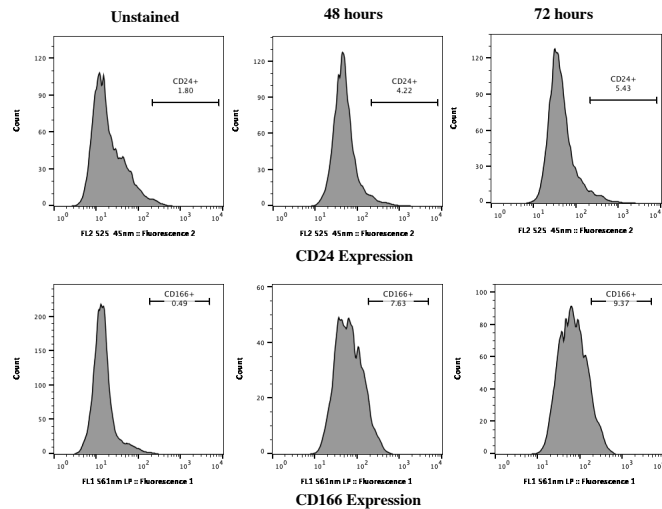
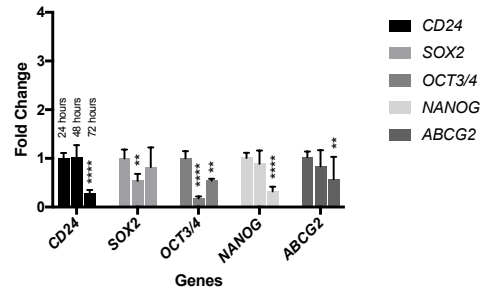
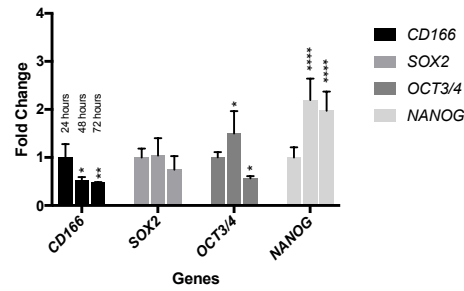


Figure 3.5: Surface protein expression of CD24 and CD166 in CD24⁻CD166⁻ sorted population in 72 hour culture. Subpopulation lacking both CD24 and CD166 subpopulations gradually recapitulate the former expression of CD24 and CD166. Values are $\bar{x} \pm SD$, $n = 3$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way Repeated Measures ANOVA was performed to investigate the effect of Time on the percentage of positive population of the respective markers.

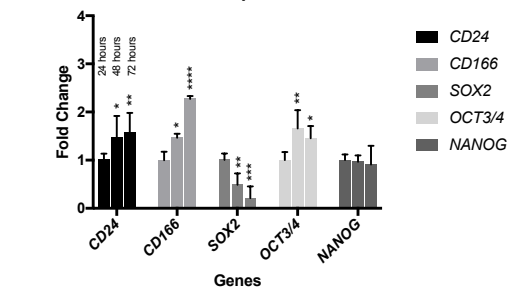
(A) CD24 Sorted Population on Stemness Genes Expression



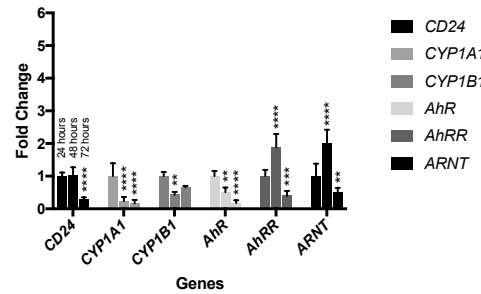
(B) CD166 Sorted Population on Stemness Genes Expression



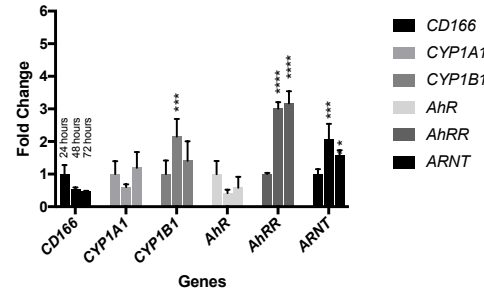
(C) CD24⁺CD166⁻ Sorted Population on Stemness Genes Expression



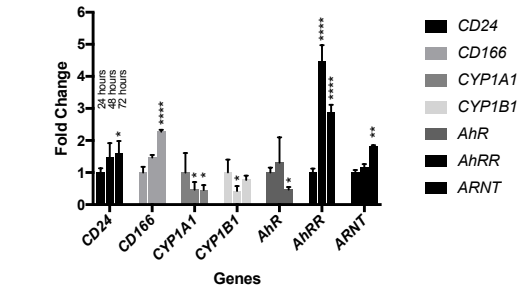
(D) CD24 Sorted Population on Xenobiotic Response Genes Expression



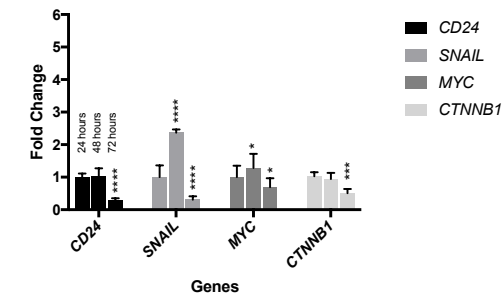
(E) CD166 Sorted Population on Xenobiotic Response Genes Expression



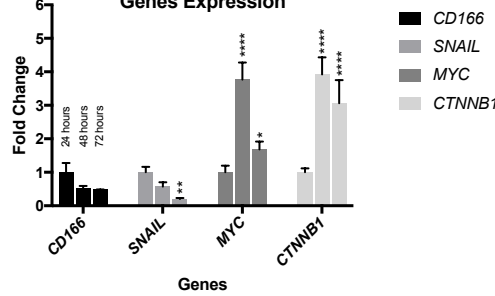
(F) CD24⁺CD166⁻ Sorted Population on Xenobiotic Response Genes Expression



(G) CD24 Sorted Population on EMT key genes



(H) CD166 Sorted Population on EMT Key Genes Expression



(I) CD24⁺CD166⁻ Sorted Population on EMT Key Genes Expression

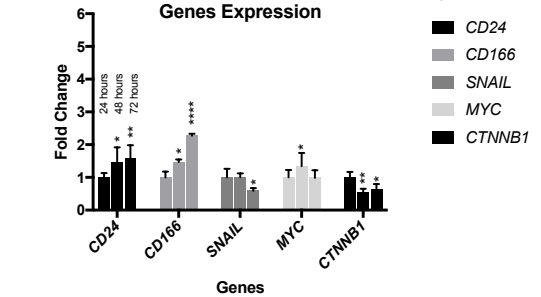


Figure 3.7: CD24⁺ and CD166⁺ subpopulation and CD24-CD166- sorted population gene expression on stemness, xenobiotic response and EMT-related genes. Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). 2-way Repeated Measures ANOVA with Gene and Time as independent variables was performed to determine whether gene expression changes with time.

3.4. Discussion

3.4.1. Is stemness expression limited to a rare subpopulation or all subpopulations of SNU-1?

Stem cell specific markers or side-population (SP) phenotypes can be used to enrich gastric CSCs [23], [41]. In the previous chapter, I found stemness factors in the unsorted SNU-1 total population; however, the signal is an average of the heterogeneous cells in the cell line. I hypothesized that sorted populations might enrich the stemness population to a threshold that can effectively be detected. Enriched populations can be beneficial to investigate specific phenotypic characteristics, especially if obtaining purer populations of interest is possible. I have shown (Figure 3.2, Table 4) that it is possible to achieve purer populations with MACS, which is consistent with the report of others [91] [237], [238]. Common stemness transcription factors such as *SOX2*, *OCT3/4*, and *NANOG* were detected in all subpopulations (Figure 3.7A, 3.7B, 3.7C). Therefore, this finding along with other findings [221], [229], [230], [232] is a paradox of the CSC model, which proposes that only rare populations of cancer cells may exhibit stemness. Neither *ALDH1* [81], [239], [240], nor another important CSC stem cell marker, *LGR5* [77], [119], [241], [242], was detected in this study. The failure to detect this marker in SNU-1 is perhaps due to its prevalence in intestinal-type gastric cancer but not diffuse-type cancer such as SNU-1 [243]. Although some studies categorized *LGR5* as one of the key CSC markers [241], [244], it is possible that the expression is more likely to be found in stem cells present in normal gastric system to maintain the gastric epithelium [145], [242], or intestinal type gastric cancer cells with poorer outcome [127] than in SNU-1 subpopulations. Notably, *NANOG* increased expression was detected in CD166⁺ subpopulation at 48 and 72 hours. While downregulation of *OCT3/4* is associated with embryonic stem cell loss of pluripotency as the cells differentiate [245], *NANOG* expression was known to suppress differentiation [246], [247]. On this basis, the pluripotency transcriptional activation of *NANOG* was seen to induce genes associated to EMT, such as in skin carcinoma [248]. *NANOG* high expression was also detected with hepatocellular carcinoma and was especially associated with its dissemination in late stages [85]. Thus, they may be possible association between the expression of *NANOG* and the EMT expression in the CD166 population (Figure 3.7B, 3.7H).

Nonetheless, *ABCG2* expression was detected only in the CD24⁺ population (Figure 3.7A). *ABCG2* is a member of ATP-binding cassette (ABC) transporter superfamily, and it is known

to contribute to cells resistance to multidrug treatment in chemotherapy [249]. It is also understood to regulate self-renewal and is a recognised stem cell marker [82], [250]. Others have reported that its expression is correlated with the degree of differentiation, and that *ACBG2* is highly expressed in poorly differentiated cells [94]. However, the decrease of expression of *ABCG2* and other stemness transcriptional factor such as *OCT3/4* in CD24 is a possible indication that the cells lose its stemness while proliferating and possibly differentiating to heterogenous progenies in the 72-hour culture. Similar effect was seen in colon cancer, where the expression of both *ABCG2* and *OCT3/4* was considerably correlated with tumour differentiation [251].

3.4.2. Do EMT-related genes express differently in the SNU-1 subpopulations?

SNU-1 is positive for β -catenin; a key molecule in Wnt/Wingless signal transduction and a key player in EMT. The gene encoded for this protein, *CTNNB1*, was detected in all sorted populations (Figure 3.7H, 3.7I, 3.7J), however its expression increased almost fivefold in CD166 population at 48 and 72 hours (when compared to 24 hours control). The promotion of EMT is made possible through activation by Wnt/ β -catenin pathway [252]. Weinberg and colleagues showed that by inducing EMT in mammary epithelial cells, the cells would acquire mesenchymal traits along with the expression of stemness markers [232]. As mentioned before, a stemness marker such as *NANOG* is among the key stemness regulators that act as an EMT inducer [253]. It does so by binding to the promoter region of EMT-associated genes such as *Snail*, or *TWIST1*, which represses *E-cadherin* and confers EMT features in skin epithelial cells [248]. As shown in the Figure 3.7B, the increase in *NANOG* expression with *CTNNB1* and *MYC* could suggest a possible crosstalk between these genes as enriched CD166⁺ cells progress in culture throughout the 72 hours. However, EMT-target gene, *E-cadherin*, cannot be detected in SNU-1 because the gene and promoter region of *E-cadherin* in SNU-1 is known to be methylated [31]. *Snail*, the gene highly associated with the EMT process [278]-[280], was detected in all subpopulations.

From the findings, it is possible to summarise that the regulation of the EMT-TFs such as *SNAIL*, *MYC* and *CTNNB1* may have different effects on the stemness expression of the three subpopulations throughout the 72 hours period after sorting. In CD24 subpopulation, EMT-TF genes were down regulated and may have possibly suppressed the overall expression of stemness genes (Figure 3.7G). However, in CD166, the repression of *SNAIL* showed minimal

effect on the suppression of stemness genes expression. While *MYC* and *CTNBB1* were upregulated (Figure 3.7H), a similar pattern of upregulation of NANOG (one of the stemness genes) was observed in CD166 (Figure 3.7B). Nevertheless, in both CD24 and CD166 sorted subpopulations, the regulation of both EMT-TFs did not prevent the differentiation of enriched subpopulations, which is hinted by the down regulation of CD24 and CD166 genes expression (Figure 3.7A and 3.7B). In the peculiar case of sorted population that lacks both CD24 and CD166 subpopulations, the down-regulation of EMT-TFs (Figure 3.7I) may possibly be linked to the increased expression of *OCT3/4*, and the upregulation of genes coding for CD24 and CD166 (Figure 3.7C), which hints at the potential dedifferentiation of the overall populations to increase the population of cells expressing CD24 and CD166 surface markers (Figure 3.5).

However, it is possible that – given the stemness expression in both CD24 and CD166 subpopulations, along with detection of *ABCG2* expression in CD24 and *CTNNB1* expression in CD166 subpopulations – these two subpopulations will play a critical role in affecting the migratory capabilities and invasiveness of the cells in the subpopulation. It is an interesting avenue for further research, based on the accumulating evidence that the activation of stemness factors could endow cells with EMT transformation [232], [233], [254].

3.4.3. Expression of xenobiotic response and drug metabolism gene in SNU-1 subpopulations

One of the key findings in this chapter is the characterisation of xenobiotic response and drug metabolism genes' expressions in the sorted population of SNU-1. Recently, *AhR* was found to control stemness expression (and thus prevent tumorigenesis in mice) [188] by the inhibition of Wnt signalling pathway *via* transcriptional gene regulation [188]. However, in the absence of an added *AhR* ligand, it is unclear from my experiments if stemness expression in this study is connected to *AhR* expression. A previously published extensive study regarding *AhR* role in stemness found that it interacts directly with *SOX2*, and *AhR* ligands can increase the interaction and cause nuclear *SOX2* translocation [239].

Moreover, findings that the heightened expression of *AhR* repressor (*AhRR*) and *ARNT* genes, and repressed level of *AhR* observed here in all isolated sub-populations, especially in the enriched CD24 and CD166 subpopulations, are confounding (Figure 3.7 D-F). These observations may yet show its importance in the regulating stemness in the cells. *AhR*

expression is transcriptionally repressed in mouse embryonic stem cells via pluripotency factors (*OCT3/4*, *NANOG*, *SOX2*) signalling pathway [255]. Yet the reversible *AhRR* expression in embryonic stem cells may affect the activation of the pluripotency factors, which in turn may activates the stem cell differentiation [255], hence giving a possible explanation for the recapitulation of the CSC marker of CD24 and CD166 in CD24⁻CD166⁻ population, or reduction of CD24⁺/CD166⁺ in their respective populations in 72 hours. Nevertheless, the role of *AhR* in a crosstalk between stemness regulation in the subpopulations of SNU-1 merits further investigations.

3.5. Conclusion

In this chapter, I suggest a possible regulatory control that can affect the stemness and phenotype of the sorted population, with *AhR* and EMT inducers playing the most prominent role. I have shown that enriched populations of known CSC markers, CD24⁺ and CD166⁺, express stemness genes, yet stemness expressions can still be identified in the population depleted of CD24 and CD166. Although the sorted population cell surface markers expression declined as the cells grew in culture, the stemness genes' expressions were still detectable. Notably, population depleted of the CSC markers, CD24⁺ and CD166⁺, seems to be producing cells expressing this marker. Regardless, the stemness factor may yet play an important role in sustaining the self-renewability of SNU-1 along with the expression of EMT markers. It is possible that EMT and stemness are both intrinsic features of CSCs but there is increasing evidence that show both factors to be parallel events [256]. Based on these findings, it is possible to conclude that the regulation of the EMT-TFs such as SNAIL, MYC and CTNNB1 affects the stemness expression of the three subpopulations throughout the 72 hours period after sorting. However, it is important to note that these effects vary by each sorted subpopulation. In general, in primary tumours, metastasis begins by acquiring the mesenchymal phenotype to initiate the EMT process [233]. Thus, acquiring 'stemness' would be a critical step, so that a phenotypic change can take place in the tumour, often by regulating embryonic pathway and master regulators of EMT-TFs such as Wnt pathway, which is controlled by key genes *CTNNB1* and *MYC* [92] and *SNAIL* [135], [163]. These observations are further supported by studies that indicated that stemness promotion is associated with EMT response [245], [246]; where tumour cells (breast cancer tumour [241], [257]-[261], ovarian cancer [237], cell lines of lung and renal carcinoma [238]-[241], mouse pancreatic and prostate cancer [242]) acquire mesenchymal-like phenotypes along with increased plasticity. Thus, this finding indicates that Wnt and EMT-TF regulators play a crucial role in controlling the cells' demography in the subpopulations (further explored in Chapter 4 and 5).

Stemness phenotype may also possibly be regulated by *AhR*, which controls cell growth by manipulating the stemness factor. Thus, it is highly likely that by inducing *AhR* activity with an exogenous ligand, it may be possible to upregulate multiple stem cell associated genes. Thus, can *AhR* activity be manipulated to influence the phenotypic expression of SNU-1? Will gene and surface protein expressions in SNU-1 change in a perturbed system by introducing *AhR* ligand in the system?

4 : Genotoxicity effect of BaP treatment to SNU-1 cells plasticity.

4.1 Introduction

In the previous chapter, I showed that gastric cancer cell line, SNU-1, expressed the CSCs surface markers of CD24 and CD166. Due to the nature and origin of SNU-1 (isolated from primary tumour, expressing stemness markers, poorly differentiated), it is an appealing choice for phenotypic change induction to a potentially plastic cell line. Benzo[a]pyrene (BaP) is one of the best studied polycyclic aromatic hydrocarbons (PAH) classified as a group 1 human carcinogen by the International Agency for Research on Cancer (IARC) [176] [174]. The mode of BaP activation has been well characterised by our research group [257]-[261]. When introduced to a cell, BaP binds to its natural ligand, the Aryl hydrocarbon Receptor (*AhR*) and forms an *AhR* receptor complex (*AhR*+BaP) [177] [178]. This complex is then carried in to the nucleus by a hsp90 chaperone complex to form a heterodimer with Aryl hydrocarbon Nuclear Translocator (*ARNT*) [179], [180]. This complex binds to the XRE binding sites (Xenobiotic response element) [181], located at the upstream regulatory region of gene targets such as cytochrome P450 such as CYP1A1 and CYP1B1 [180], [182] to transcribe necessary enzymes for the metabolism of BaP. The carcinogenic byproduct B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE) can covalently bind DNA guanine residues to form pre-mutagenic adducts [183], [184]. In this manner, BaP can promote cell proliferation and metastasis through *AhR* [177], [185], yet the genotoxicity potential of BaP is lost in mice that lack the *AhR* receptor [186]. *AhR* activation by dietary ligands is reported to contribute to stem cell niche maintenance in stomach and can restrict intestinal stem cells growth by inhibiting Wnt/ β -catenin-signaling pathway [188]. This shows that *AhR* can also actively impact the stemness of a cell. As previously explained in Chapter 1 (see section 1.8 on EMT), epithelial-mesenchymal transition (EMT) plays a crucial role in cancer progression, especially in metastasis [253]. This process also triggers production of CSC-like cells [262] through the activation of Wnt/ β -catenin-signaling pathway [263]. In some cells with partial-EMT characteristics (co-expressing epithelial and mesenchymal characteristics) [264] [265] [266], [267], high stemness expression was detected and stemness characteristics such as plasticity increase, tumorigenic potential and invasiveness [195], [217]. [268]. In line with the previous findings, in this chapter I am interested in investigating the effect of molecular response to SNU-1 plasticity when treated with BaP.

4.1.1 Hypotheses:

- Molecular genotoxicity mediated by BaP and *AhR* can induce plasticity in the gastric cancer cell line SNU-1.
- *AhR* activation will also lead to SNU-1 phenotypic change to increase invasiveness and migratory capacity, CSC marker expressions, and stemness factors.

4.2 Materials and Methods

4.2.1 Cell culture

Human gastric cancer cell line SNU-1 was purchased from American Type Culture Collection (ATCC) and cultured as recommended by ATCC. SNU-1 was routinely cultured in ATCC RPMI-1640 Medium (ATCC® 30-2001™) supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin (GIBCO, Life technologies) in T75 flasks. Cells were incubated at 37°C in a humidified incubator (5% CO₂).

4.2.1.1 Cell treatments

Cells were seeded at a density of 1×10^5 cells per well in a 24 well plate. For treatment with dietary carcinogen, cells were treated with BaP (0- 10 µM, Sigma-Aldrich). The BaP chemical was dissolved in DMSO (Dimethyl sulfoxide, Sigma-Aldrich), and a negative control (treatment without adding any BaP; annotated as “0” in the graphs), made of 0.2 % DMSO in distilled H₂O, was used in all treatments. Early passage cells were used for all experiments (passage 7-10).

4.2.2 Flow cytometry analysis

Labelled fraction and unlabelled fraction of cells from magnetic separation were processed for flow cytometry analysis. Firstly, cell counts were performed using an automated cell counter machine (Moxi Z from ORFLO Technologies), and 1×10^6 cells were added into each FACS tube and incubated on ice for 15 minutes. New medium (1 ml) was added to as many tubes as required and centrifuged for 500×g for 5 minutes. PBA (30 µl of 1% Bovine serum albumin dissolved in Phosphate buffered solution) containing 3 µl of FcR Blocking reagents (Miltenyi Biotec) was added to each tube containing the cells to reduce non-specific binding of the antibodies. PBA also stabilizes the cell membrane and prevents antibodies from sinking into the membrane.

Next, supernatants were discarded, and 10 µl of an antibody of each CD markers (refer to Table 4.1 for concentrations) diluted in 100 µl of staining buffer were added to 1×10^6 cells' pellet in each respective tube labelled with the corresponding CD marker being used. Then the tubes were incubated for 30 minutes on ice. It is worth noting that antibodies were titrated to

determine optimal antibody concentration for live-cell labelling to achieve the best discrimination between positive and negative cells, while keeping nonspecific antibody binding to a minimum, according to the protocol's recommendation [210]. Finally, 500 μ l PBA was added to each tube, and the samples were centrifuged for 400 $\times$ g for 15 minutes, and the supernatant was discarded to remove excess antibodies. Cell pellets were resuspended in 1 ml of PBA and were examined with flow machine (BD FACS Fortessa). The results were analysed using FlowJo software, version 10.1r1, on Mac OS X El-Capitan Operating System, version 10.11.5.

Table 4.1 CD markers used in the experiments:

CD Markers	Supplier	Supplier items code	Concentration (per 10 μ l)
FITC Mouse Anti-Human CD24	Becton Dickinson	555427	0.2ug/ μ l
PE Mouse Anti-Human CD166	Becton Dickinson	559263	0.0125ug/ μ l

Fluorescence Minus One (FMO) Control) is used in this experiment. The FMO control is employed to correctly interpret flow cytometry data, and especially in the case in which the population of interest is meagre; it acts as a reasonable quality control. It is used to identify and gate cells in the context of data spread due to the multiple fluorochromes in each panel. An FMO control contains all the fluorochromes in a panel, except for the one that is being measured. Therefore, since there were only two antibodies used, the FMO for each sample would be the unstained sample without any antibody.

Table 4.2 Experimental design with FMO for SNU-1 cells.

Antigen	FITC	PE
CD24 FMO	-	CD166
CD166 FMO	CD24	-

4.2.3 Cell Viability Assay

Cell growth was determined by PrestoBlue® Cell Viability Reagent (Thermofisher). This cell permeable resazurin-based solution (PrestoBlue®) indicates the cell viability *via* quantitative measurement of the proliferation of cells by assessing the reducing power of viable cells. When added to cells, the PrestoBlue® reagent is modified by the reducing environment of the viable cell and turns red, becoming highly fluorescent. SNU-1 (ATCC RPMI-1640 Medium) was plated into 24-well plates at cell number: 2.5×10^4 , 5×10^4 , 1×10^5 , 1.5×10^5 , 2×10^5 , and 2.5×10^5 . Cells were incubated for 24, 48 and 72 hours, and 1 ml of PrestoBlue was added to each well and further incubated for 30 minutes respectively for each time point per manufacturer's recommendation. The fluorescence was recorded on a microplate reader (Synergy Hybrid, Biotek) at a wavelength of 535 nm (excitation) and 615 nm (emission). Top read and bottom read format were recorded, due to the different adherence nature of the cells.

4.2.4 Gene Expression Studies

4.2.4.1 RNA extraction

RNA was extracted from at least 1×10^6 cells using the RNAeasy kit (Qiagen). The kit is supplied with Qiagen's proprietary RLT lysis buffer, RW1 RNA precipitation buffer, and RPE RNA washing buffer. Harvested cells were centrifuged at $1500 \times g$ for 5 minutes to pellet the cell. RLT buffer plus (a lysing buffer; added 10 μ l β -Mercaptoethanol (B-ME) to 1ml RLT buffer) was added to the pelleted cells (350 μ l for $< 5 \times 10^6$ cells / 600 μ l for $< 1 \times 10^7$ cells), and vortexed for 30 seconds. The lysate was centrifuged for 3 minutes at maximum speed ($18000 \times g$). The supernatant was removed by careful pipetting, and the lysate was transferred to the genomic DNA Eliminator spin column that came with the RNAeasy kit and placed in a 2 ml collection tube to remove DNA contaminations. Columns were centrifuged for 30 seconds at $8000 \times g$. Columns were discarded, and flow through supernatant (contained RNA) from the centrifugation was kept aside. RNA precipitation was done by adding 1 volume of 70% ethanol (same volume as RLT volume amount used, *e.g.* 350 μ l/600 μ l). Next, up to 700 μ l of samples transferred to RNAeasy spin column were placed in a 2 ml collection tube, and centrifuged for 15 seconds at $8000 \times g$, and flow through was discarded (the supernatant from the centrifugation does not contain RNA, instead RNA is retained in the RNAeasy column). RNA in the RNAeasy spin column was washed with 700 μ l of RW1 buffer, followed by washing with 500 μ l of RPE buffer; both times centrifuged at $8000 \times g$ for 15 seconds. Precipitated RNA was washed for the

final time with 500 µl RPE buffer, centrifuged for 2 minutes at 8000×g. For RNA collection, RNAeasy spin columns were placed in a new 1.5 ml collection tube, and 30 µl of RNAase-free water was added to elute the RNA into collection tubes. Samples were centrifuged for 1 minute at 8000×g. Optionally, the column may be spun again with another volume of water, or the RNA elute to maximise RNA collection. Finally, RNA extracts in each tube were labelled accordingly and quantified by UV spectroscopy (UV-VIS Nano-spectrophotometer, Implen, Essex, UK) with purity assessed from 260/280nm and 260/230nm ratios. Extracts were stored at -80°C until used.

4.2.4.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

cDNA strands were synthesized by using a reverse transcriptase kit, Superscript II enzyme (Thermofisher). RNA extracts (100-500 ng) from each sample were added to 300 ng of random primers, heated for 5 min at 65°C and then quickly placed on ice. Each tube (containing the RNA extracts and random primers) was incubated with 0.5 mM dNTPs, 1x first strand buffer, 8µM dithiothreitol and 100 units of Superscript II reverse transcriptase (Invitrogen, Life technologies) for 10 min at 25°C, 90 min at 42°C and 15 min at 70°C in a thermocycler (Peltier Thermal Cycler PTC-200, MJ Research, Waltham, Massachusetts, USA). An mRNA reverse transcription kit was used for RT reaction according to the manufacturer's protocol (Taqman, Applied Biosystems, Life Technologies). Quantitative-Polymerase Chain Reaction (qPCR) was performed using Taqman probe pre-designed gene expression assays (as detailed in Appendix A) and FAST PCR master mix (TaqMan, Applied Biosystems, Life Technologies), and measured in a StepOnePlus fast real-time PCR system (Applied Biosystems, Life Technologies) according to the manufacturer's protocol. GAPDH was used to normalise cellular mRNA expression respectively (Appendix A). Gene expression was quantified using the delta-CT method.

4.2.5 Invasion and Migration Assay

4.2.5.1 Invasion assay

Matrigel invasion chambers were obtained from Corning (USA, catalogue number: 354480). The cell culture inserts containing 8-micron pore size PET membraned had been pre-treated with Matrigel Matrix by the manufacturer and could be placed into a standard 24-well plate. The chambers were placed in a 24-well plate, and 500 µl of warm RPMI culture medium were added to the interior of the chambers, and another 500 µl of the medium were added to the well to moist the exterior of the chambers. After 2-hour rehydration the medium was removed from the chambers and was ready to use for an invasion assay. To perform the invasion assay, hydrated chambers were placed on the 24-well plate, with 500 µl of RPMI culture medium supplemented with 1% dextran-coated charcoal-stripped FBS added in the Matrigel chambers, and 500 µl of culture medium with 10% FBS were added at the bottom well (where the chambers are placed) to act as a chemoattractant. 1×10^4 cells were plated into each chamber. Then, five concentrations (1×10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} M) of BaP were added into the chambers and left in cell incubator for 48 and 72 hours. After the incubation period, media in the Matrigel chambers cells were removed, and the cells attached to the Matrigel were gently removed with a cotton swab. For further analysis of the Matrigel with GIEMSA staining, Matrigel chamber was washed twice with the PBS solution to remove any residual cotton or cells. The cells were fixed by adding 500 µl formaldehyde (3.7% in PBS; Sigma Aldrich), and left in room temperature for 2 minutes. Then chambers were washed twice with the PBS solution for the second time. The cells embedded in Matrigel were permeabilised by adding 500 µl of 100% methanol (Sigma Aldrich) into the chambers and left in room temperature for 2 minutes before being washed twice with the PBS solution for the third time. Finally, the cells were stained with 250 µl Giemsa Stain (Stock solution: 3.8g Giemsa powder (Sigma) in 250 ml methanol and 250 ml glycerol. Working solution: 10 ml of stock solution added to 80 ml of distilled water and 10 ml of methanol) and incubated for 15 minutes in room temperature. Plate containing the Matrigel chambers stained with Giemsa was covered with aluminium foil to prevent any light exposure. Finally, the solution was removed, and chambers were washed twice with PBS. Cotton swabs were used to remove non-migrated cells from the Matrigel surface. Stained cells were viewed under Leica SP8 inverted microscope (Leica, Germany).

4.2.5.2 Migration assay

For migration assay, 1×10^4 cells per well were plated in the upper chamber of a 96-transwell insert system with an 8 μm pores PET membrane (no Matrigel layer; BD Falcon, Oxford, UK) and with 500 μl of RPMI culture medium supplemented with 1% dextran-coated charcoal-stripped FBS. Meanwhile, 500 μl of culture medium with 10% FBS were added at the well (where the chambers are placed) to act as a chemoattractant. Similarly, to the invasion assay, five concentrations (1×10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-6} M) of BaP were added into the chambers and left in cell incubator for 48 and 72 hours. In both cases, for invasion and migration assay, the number of cells migrated into the well was counted with Moxi Z cell counter (Orflo, USA). Results are expressed as the number of cells migrated to the lower chamber.

4.2.6 Micronucleus assay

Micronucleus (MN) assay was performed according to OECD guidelines and based on the modified protocol established in our laboratory [259], [260]. 2×10^4 cells were seeded per well of a 24-well plate for 2D cell culture and grew in stripped FBS RPMI for 72 hours. Stripped FBS RPMI medium was removed from the 24 well plate, and the cells were washed with PBS. Then, the cells were treated with various concentrations of BaP (1×10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-6} M) and cultured for further 48 hours with 10% FBS prior to harvest. Etoposide (Sigma-Aldrich) was used as a positive control in all assays at a concentration of 125 nM as it is a known inducer of MN formation. Cells were then harvested and resuspended in culture medium containing 2% pluronic (GIBCO, Life technologies). Cells were fixed with 100% methanol onto microscope slides at a density of 2×10^4 cells per slide and stained for 60 seconds with acridine orange (0.1 mg/ml dissolved in PBS, Sigma-Aldrich). Frequency of MN was scored randomly in 1000 cells per sample and four biological replicates were performed per treatment.

4.2.7 Statistical Analysis

Data were obtained from measurements made in at least four biological replicates and presented as a mean value, $\bar{x} \pm$ Standard deviation (SD). Raw data were recorded and organized in Excel 2016 Microsoft Office program. The statistical analysis was conducted in GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA). The normal distribution of the data sets was checked using the Shapiro-Wilk normality test. Then significant differences ($p < 0.05$) were determined using one-way analysis of variance (*ANOVA*) followed by a Dunnett post-hoc test, or a linear trend analysis and two-way *ANOVA* followed by a Bonferroni post-hoc test. Pearson's correlation coefficient test was used for the correlation analysis.

4.3 Results

4.3.1 Does BaP affect SNU-1 cell proliferation?

At 48 hours, BaP induced SNU-1 proliferation at $1 \times 10^{-5}\text{M}$, $1 \times 10^{-6}\text{M}$, and $1 \times 10^{-7}\text{M}$ BaP concentrations (Figure 4.1). The increase in the cell number is indicated by the PrestoBlue™ fluorescence intensity value.

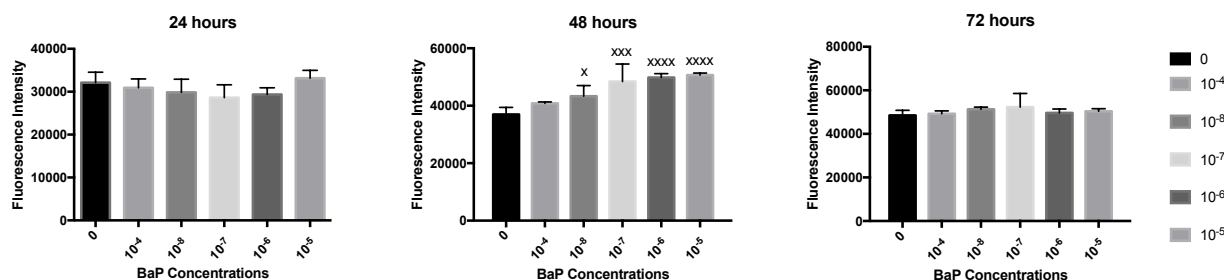


Figure 4.1: Fluorescence intensity represents the viable SNU-1 cells during treatment with BaP. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm \text{SD}$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed to investigate the effect of various BaP concentrations on the increase of the cell number (indicated by the fluorescence intensity value).

4.3.2 AhR activation and CYP1 family regulation in SNU-1

CYP1A1 and *CYP1B1* expressions are regulated by the *AhR*, a ligand activated transcription factor. Notably, *AhR* showed significant induction with the highest BaP concentration ($1 \times 10^{-5}\text{M}$; Figure 4.2). As a result, *CYP1A1* and *CYP1B1* expressions increased at the highest dose treatment too. Within 48 hours, *AhR* and *CYP1B1* were co-expressed in a dose-dependent manner. However, by 72 hours, the induction effect was lost in both *AhR* and *CYP1B1* as expression levels equalized, whereas *CYP2E1* expression was not induced. Overall, this finding suggests that the *AhR* and *CYP1* family gene expression can be activated by BaP treatment (Figure 4.2). QRT-PCR of *AhR* and *ARNT* genes expression also showed that both genes were considerably induced with the BaP treatment in a dose-dependent manner (Figure 4.3).

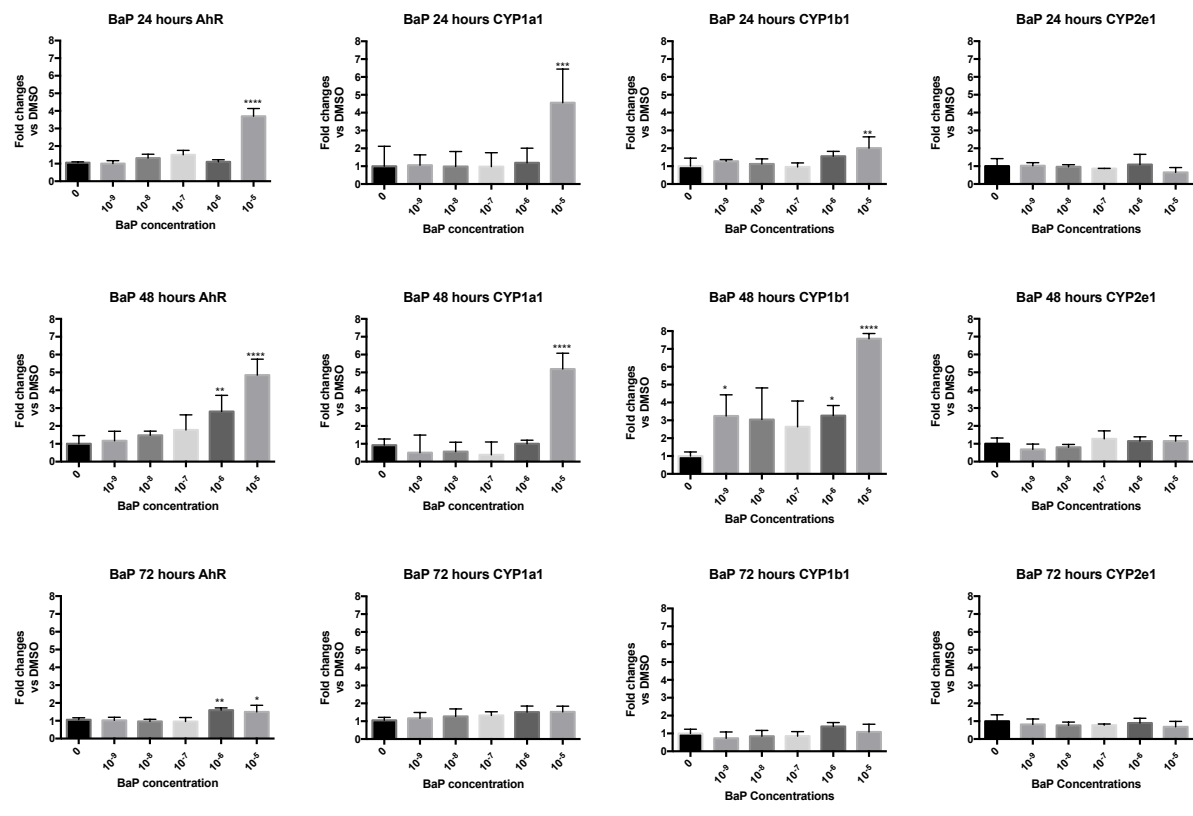


Figure 4.2: AhR and CYP1 family enzyme gene expressions when treated with various doses of BaP throughout 72 hours' culture. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed to investigate the effect of BaP concentration on the expression of AhR and CYP1 family enzyme genes.

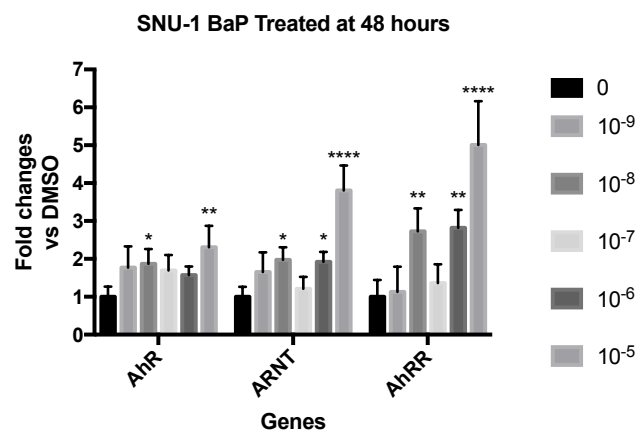


Figure 4.3: AhR, ARNT and AhRR expressions when treated with various concentration of BaP after 48 hours culture. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). 2-way ANOVA with Gene (AhR, ARNT, and AhRR) and BaP concentration as independent variables was performed to determine whether gene expression changes with BaP concentration.

4.3.3 Formation of micronuclei in SNU-1 resulting from BaP treatment, a known genotoxic compound

As a pilot test, I conducted a preliminary investigation with only two doses of BaP, $1 \times 10^{-5}\text{M}$ and $1 \times 10^{-8}\text{M}$, to inspect possible types of genetic damage at 72 hours (Figure 4.4, left graph). The positive control used in the experiment, etoposide, caused a significant elevation of micronuclei (MN), binuclei (BN) and micro-binucleus (MNBN) formation (Figure 4.5). Similarly, treatment with BaP caused similar genetic damage to the cells, although with much lower frequency. Based on this trial-experiment, I conducted a new study with a larger dose-range of BaP treatment at 72 hours (Figure 4.4, right graph). Notably, with the increase in BaP concentration, the higher number of cells with some form of nuclear damage was found. This finding prompts the question of whether the biological consequences of BaP treatment could be responsible phenotypic change of stemness markers.

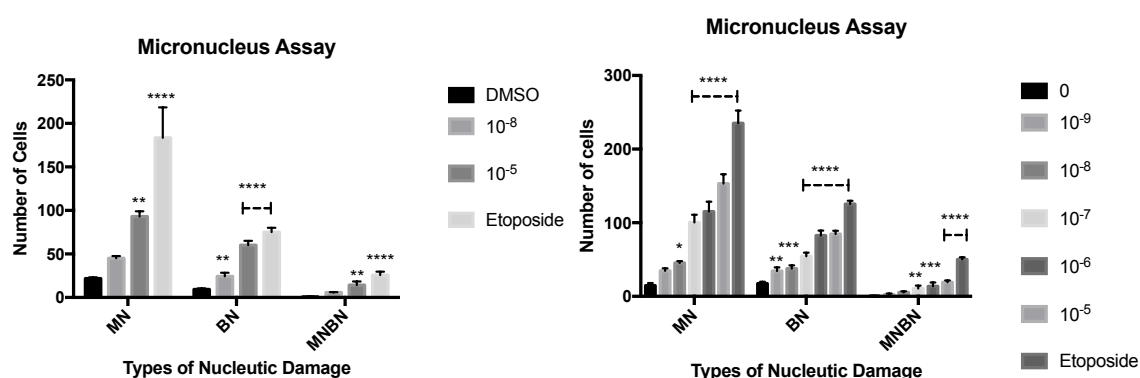


Figure 4.4: (Left graph) Preliminary data on micronucleus assay with 2 different concentrations of BaP at 72 hours. (Right graph) Micronucleus assay with BaP treatment with 5 different concentrations at 72 hours. Etoposide was used as a positive control in both assays. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm \text{SD}$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). 2-way ANOVA with Types of Nucleic Damage (MN, BN, and MNBN) and BaP concentration as independent variables was performed to determine whether BaP concentration affects the frequency of the formation of the nucleic damage in the cells.

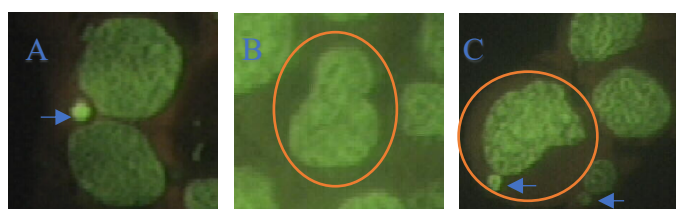


Figure 4.5: (A) micronucleus (MN), (B) binucleus (BN), and (C) micro-binucleus (MNBN) formation in SNU-1. Blue arrows indicate the micronucleus, whereas orange circles indicate binucleated cells.

4.3.4 Does BaP treatment affect the transcriptional regulation of stemness regulatory elements: OCT3/4, SOX2, and NANOG?

Notably, P trend¹¹ analysis showed that the increased induction was detected in all three stemness genes in a dose-dependent manner. The highest induction effect was found with the highest BaP concentration, 1×10^{-5} M (Figure 4.6).

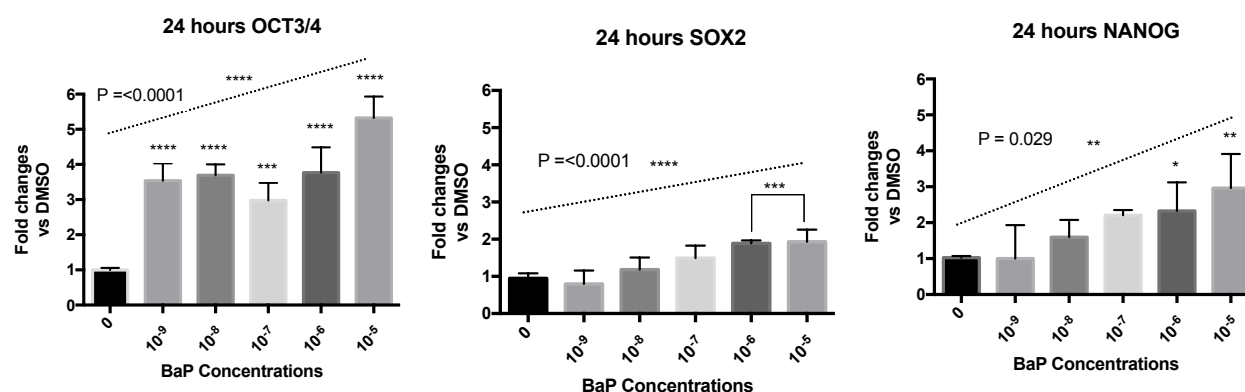


Figure 4.6: Stemness gene Sox2, Nanog and Oct3/4 expressions were evaluated with different doses of BaP. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed to investigate the effect of BaP concentration on the expression of the stemness genes.

4.3.4.1 Does these BaP-mediated stemness genes' expression change over time?

The stemness genes' expressions varied during the 72-hour treatment with BaP (see Figure 4.7). Higher fold-change of *SOX2* at 24 hours, and *NANOG* mRNA expressions was detected at 24 and 48 hours, whilst effect gradually decreased at 72 hours. Positive BaP-induction effect to *OCT3/4* level of expression were detected at 24 and 72 hours. In summary, when compared to the negative control (DMSO treated only, without BaP), the increased fold-changes with BaP treatment are a strong indication of the chemical induction effect to the phenotypic expression of the stemness markers in SNU-1. Next, I investigated the effect of BaP on the SNU-1 CD24 and CD166 subpopulation surface marker expressions.

¹¹ P trend is a statistically significant trend. The trend takes into account all of the data and can be significant even when the individual points are not significant.

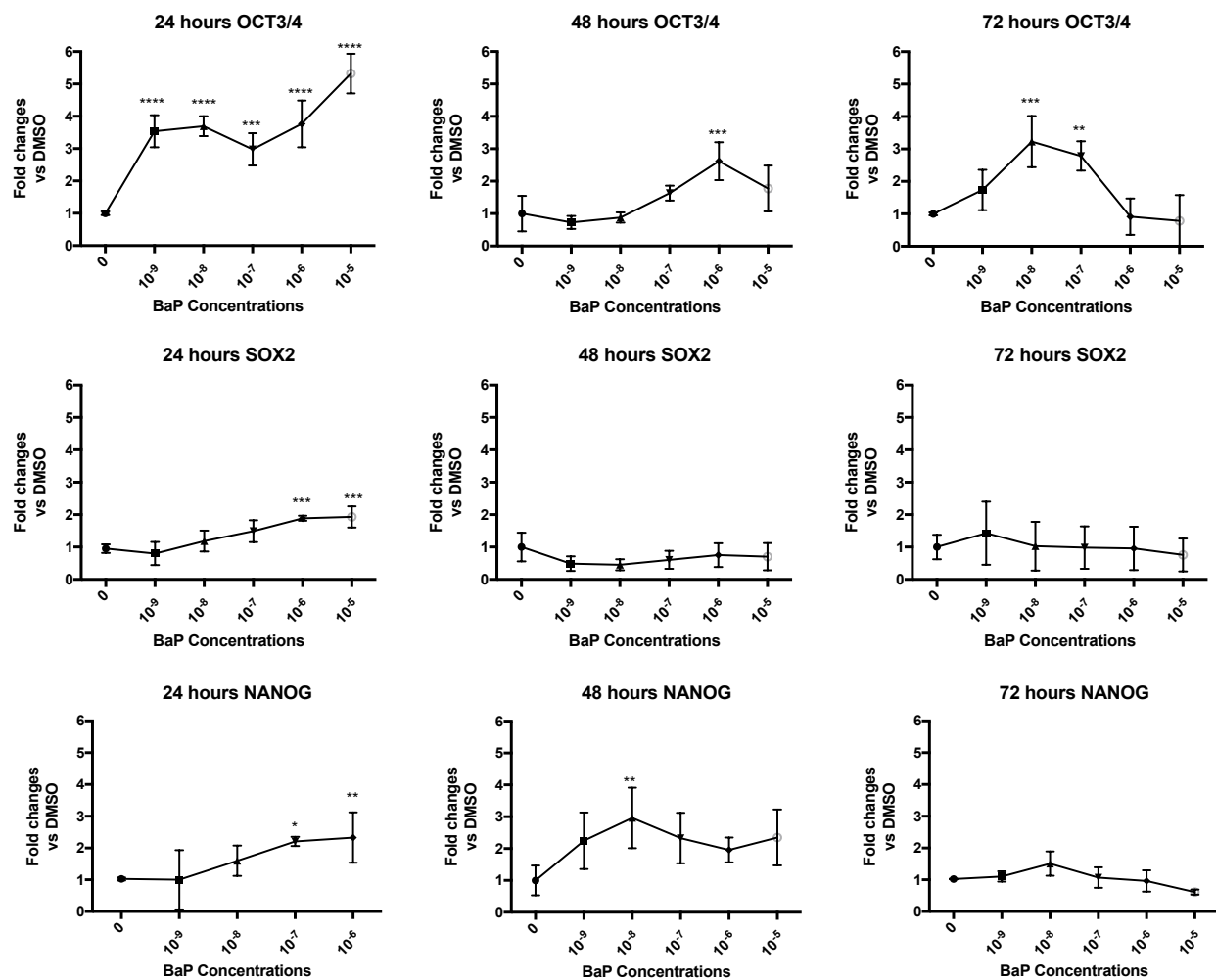


Figure 4.7: Stemness gene SOX2, NANOG and OCT3/4 expressions were evaluated with different doses of BaP at different time points. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed to investigate the effect of BaP concentration on the expression of the stemness genes.

4.3.5 Does BaP affect the protein expressions of sub-populations of CD24 and CD166 in total SNU-1?

SNU-1 cells showed the increase in the number of cells with CD24 surface marker expression with BaP treatment. Throughout 72 hours, negative control (DMSO treated) samples showed no significant increase in CD24 surface marker expression, maintaining the range of 7-9% of CD24⁺ (see Figure 4.8). However, BaP induced phenotypic change in the SNU-1 cells CD24 expression, with optimal effect at 48- and 72-hours incubation.

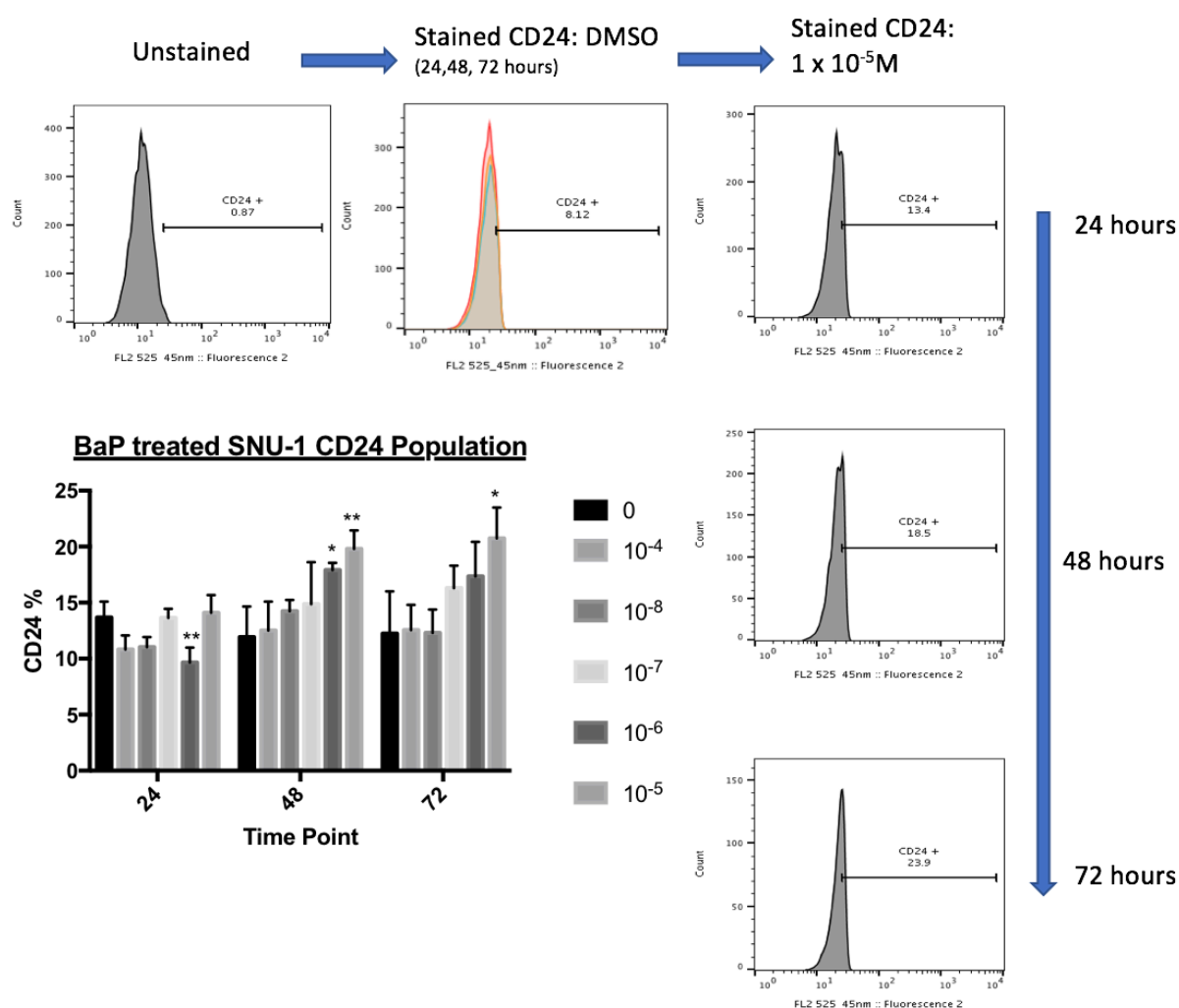


Figure 4.8: SNU-1 was treated with various concentrations of BaP, then checked for CD24 marker expression over 72 hours' treatment. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Two-way Repeated Measures ANOVA with Time and BaP concentration as independent variables was performed to check whether BaP concentration has an effect on the percentage of CD24 population in SNU-1 during 72 hours' treatment.

Similar increase in CD166 surface marker expression was found in treatment with higher BaP concentrations. Throughout 72 hours, the negative control (DMSO treated) samples showed little change in the CD166 surface marker expression, maintaining the range of 10-15% of CD166⁺ (Figure 4.9). This data showed that BaP can also induce phenotypic change of the SNU-1 cells CD166 expression. This data also suggests the potential plasticity of SNU-1 cells as the BaP can induce phenotypic change to both subpopulations at 48 hours (Figure 4.10). However, does SNU-1's phenotypic response to BaP treatment affect changes in EMT genes' expressions?

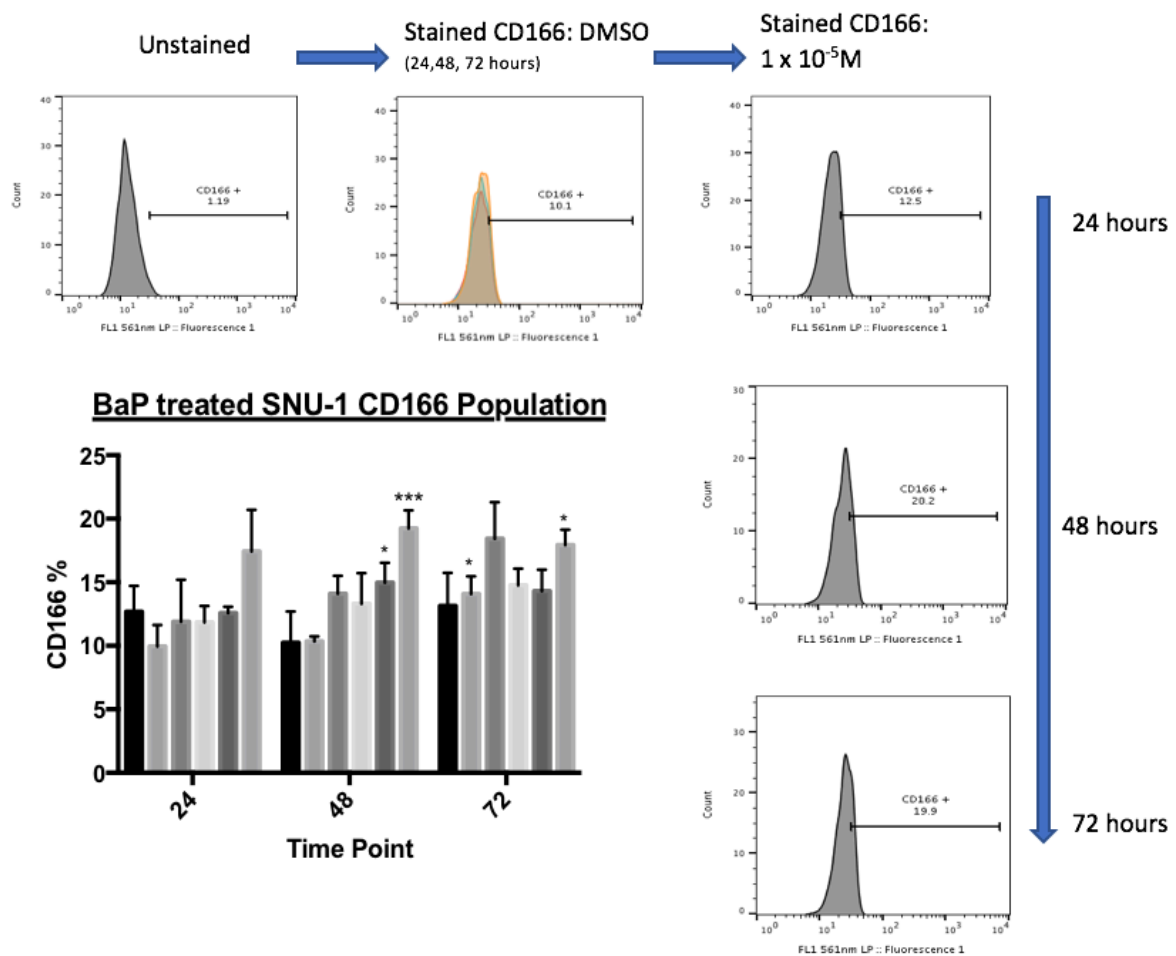


Figure 4.9: SNU-1 was treated with various concentrations of BaP, then checked for CD24 marker expression over 72 hours' treatment. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Two-way Repeated Measures ANOVA with Time and BaP concentration as independent variables was performed to check whether BaP concentration has an effect on the percentage of CD166 population in SNU-1 during 72 hours' treatment.

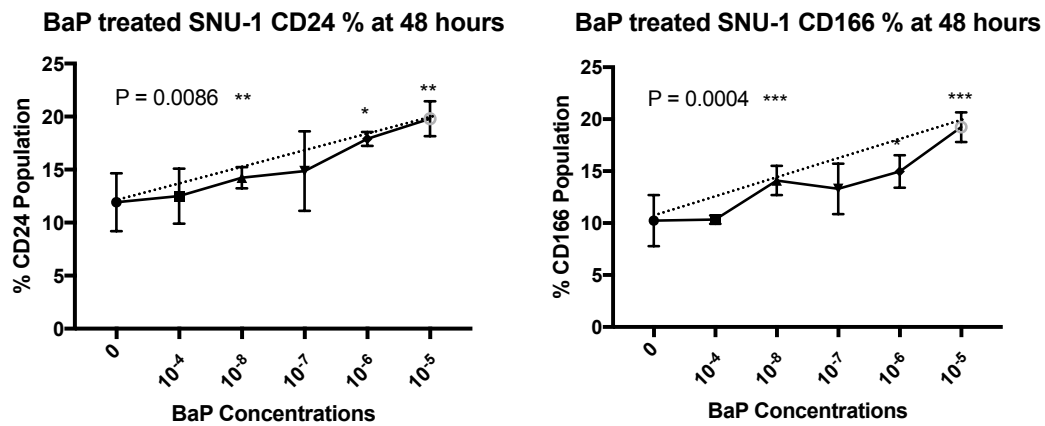


Figure 4.10: SNU-1 CD24⁺ and CD166⁺ expression at various concentration of BaP at 48 hours. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed to investigate the effect of BaP concentration on the positive expression of CD24 and CD166 in SNU-1, respectively, at 48 hours.

4.3.6 BaP effect on EMT-inducer genes expression in SNU-1

Snail (or *SNAIL*) is one of the key transcription factors involved in regulating EMT. Wnt/ β -catenin signalling pathway is also necessary to initiate the stemness and plastic, mesenchymal-like phenotype during the transition. With BaP treatment, Snail gene expression was induced at 48 and 72 hours. Notably, the highest induction effect was found at the 72 hours incubation (Figure 4.11). This finding suggests that BaP may potentially induce SNU-1 cells to a mesenchymal state, potentially inducing the increased level of cell motility (see Section 4.4.6.1).

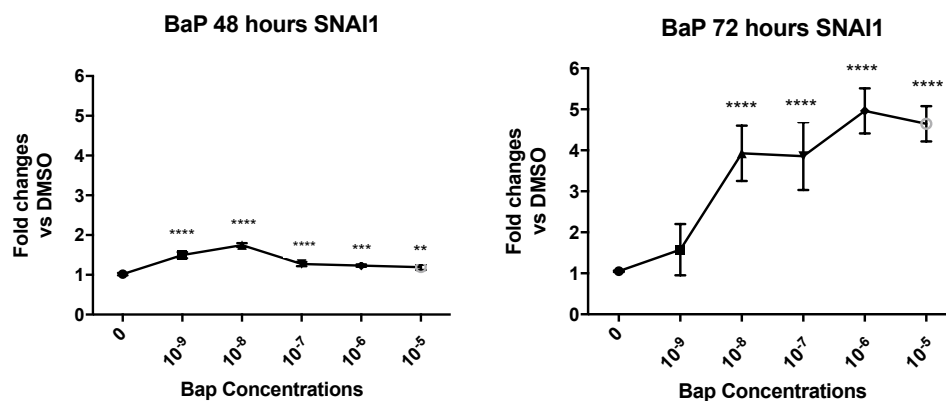


Figure 4.11: Snail gene expressions were evaluated with QRT-PCR on BaP treated SNU-1 cell population at 48 and 72 hours. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed to investigate the effect of BaP concentration on the expression of SNAI1, at 48 and 72 hours, respectively.

The expression of a key gene in Wnt signalling pathway, *CTNNB1* gene, was significantly induced with BaP treatment at higher concentrations at 72 hours compared to DMSO treated samples (Figure 4.12). Similarly, BaP appeared to promote the same induction effect to *MYC* gene expression (Figure 4.12). On the other hand, *SHH* gene was also tested but not detected in any sample.

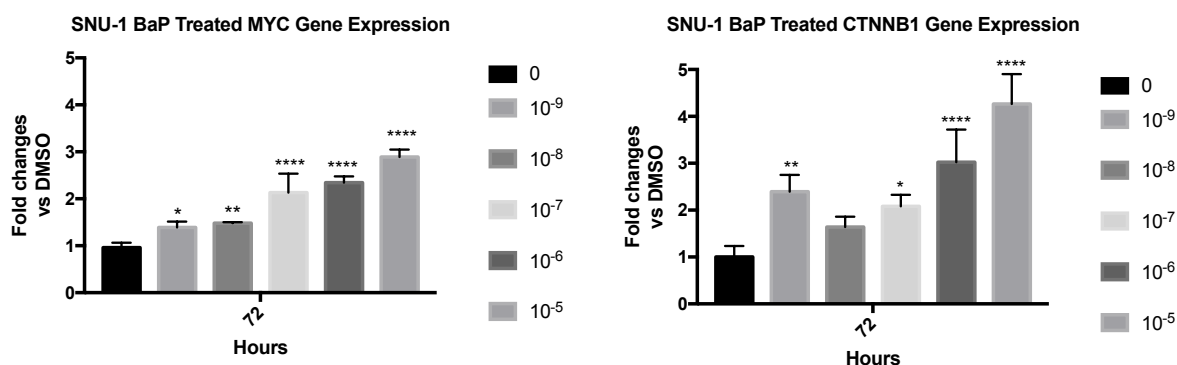


Figure 4.12: *MYC* and *CTNNB1* gene expressions treated with various BaP concentration for 72 hours. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed separately to investigate the effect of BaP concentration on the expression of *MYC* and *CTNNB1*, at 72 hours.

4.3.6.1 BaP treatment effect on SNU-1 invasion and migratory ability

To further test my hypothesis on BaP induced plasticity can induce migrative and invasive transformation of the SNU-1 cells, I performed series of cell invasion and migration assay. The Invasion assay would determine the ability of the cells to digest and move through the Matrigel (extracellular matrices) by counting the number of cells that moved into the bottom well, whilst migration is the measure of the potential of migratory reaction of endothelial cells assessed by the number of cells traversing a porous membrane (no Matrigel).

Interestingly, at 72 hours (see Figure 4.13), significant number of cells migrated and invaded through the Matrigel. The observed patterns of migration and invasion of the SNU-1 treated cells are remarkably similar to the expression of *Snail* treated with BaP.

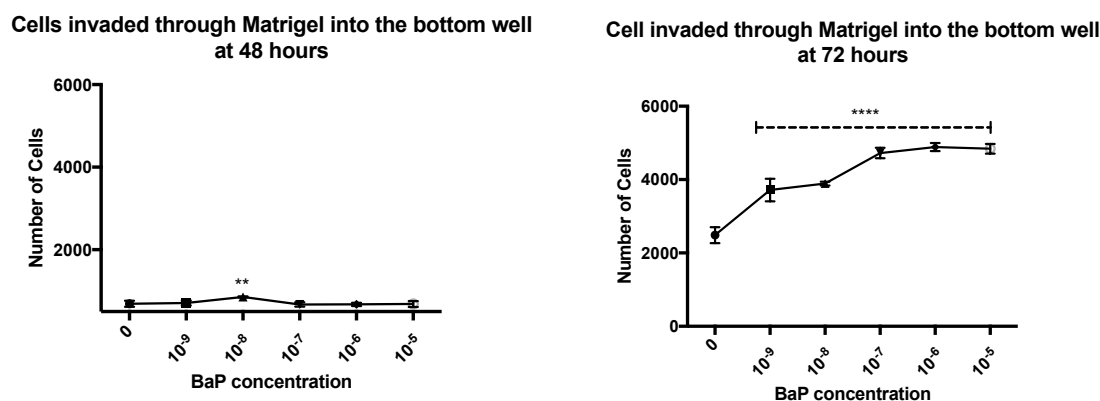


Figure 4.13: SNU-1 cells treated with BaP at 48 and 72 hours cultured in Trans-well migration assay with Matrigel™. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed separately to investigate the effect of BaP concentration on the invasiveness of the treated cells, at 48 and 72 hours.

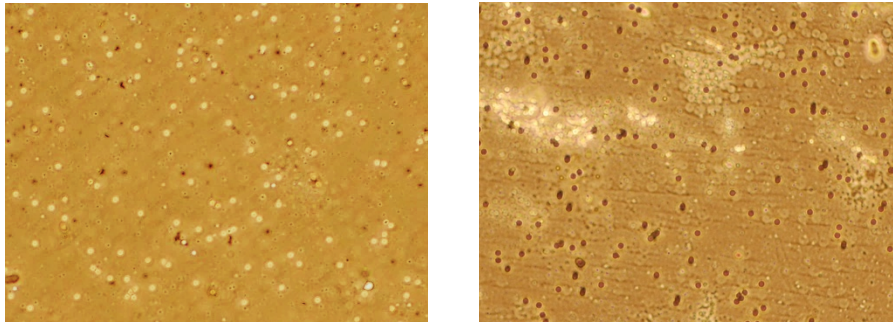


Figure 4.14: Matrigel embedded inside the Trans-well and media removed. Image (x10) captured at the middle coordinate/centre zone of the well. (Left: BaP dose 1×10^{-5} at 48 hours, Right: BaP dose 1×10^{-5} at 72 hours).

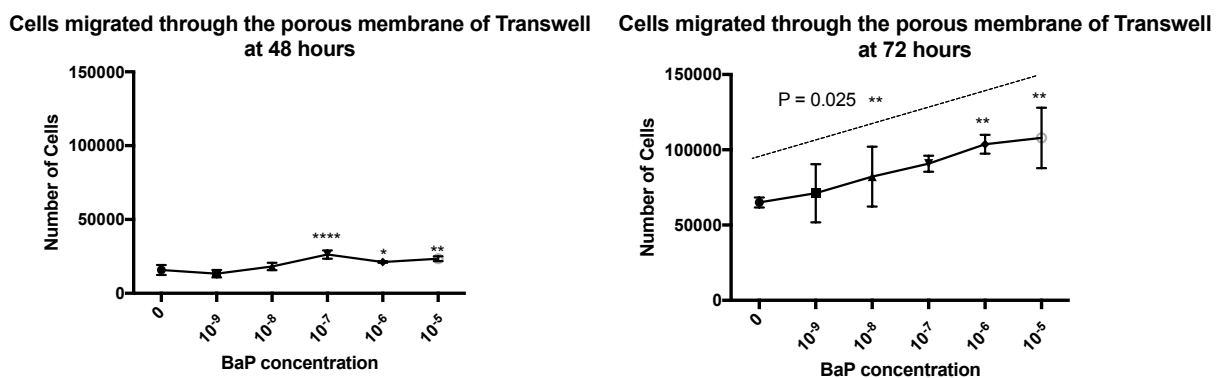


Figure 4.15: SNU-1 cells treated with BaP at 48 hours cultured in Trans-well migration assay (without Matrigel). Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed separately to investigate the effect of BaP concentration on the migratory ability of treated cells, at 48 and 72 hours.

To further confirm this finding, I repeated the experiment at 48 hours with a different analyzing method to confirm whether the cells were also actively invading the gel at this time-point, or whether migratory and invasion effect could only occur at 72 hours. Rather than counting the cells that penetrated the Matrigel, Matrigel was isolated from the wells, cleaned, and treated with Giemsa to stain the cells retained in the gel. Stained Matrigel was captured by high resolution wide-field microscope, then analyzed with Fiji software, Image J to calculate the number of identified purple spots (refers to the stained cells with Giemsa solution¹²; Figure 4.16). Significant numbers of cells were found to invade the Matrigel at all dose concentrations of BaP (Figure 4.17). The finding indicates that BaP treated SNU-1 cells actively penetrate the Matrigel, which means that earlier exposure to BaP can also induce change in the aggressiveness of the cell behaviour.

¹² One of the best-known histological stains, Giemsa stain colours the nuclei in dark blue and the cytoplasm to pink, depending to the acidity of the cytoplasmic contents.

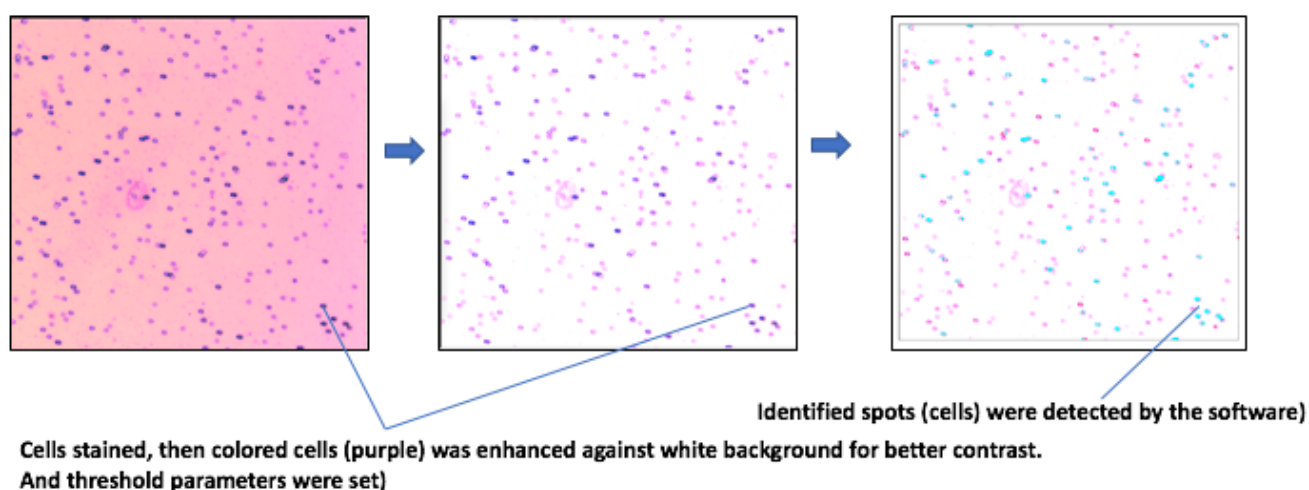


Figure 4.16: Selection criteria of the stained cells on the Matrigel using Fiji Software from captured images. This method calculates the number cells that are actively invading the membrane of Matrigel.

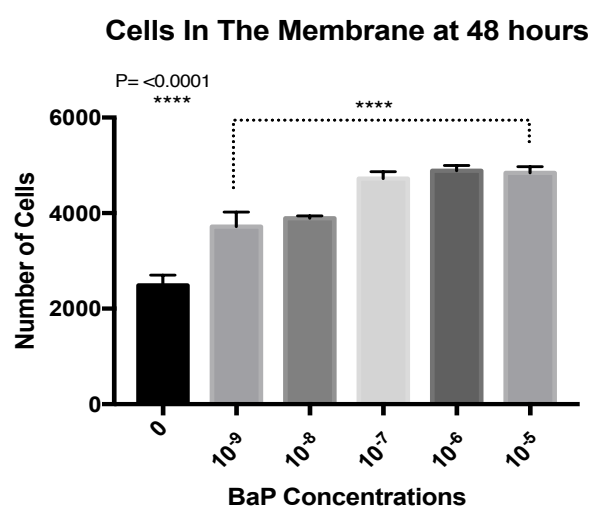


Figure 4.17: The number of treated SNU-1 cells indicating cells stained while passing through the Matrigel. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed to investigate the effect of BaP concentration on the invasiveness of the treated cells, at 48 hours.

4.3.6.2 Effect of BaP treatment on invading SNU-1 total cell population surface protein expression.

Another setup of the invasion assay was conducted to investigate the effect of BaP treatment on the invading SNU-1 cells surface markers at 48 hours. Almost 30% of invading cells were CD24⁺, whereas ~20% were CD166⁺ (Figure 4.18). This finding suggests a possible link between the induction of EMT with BaP treatment of the invading subpopulations. However, it is still unclear if BaP effect on the observed phenomenon results from the BaP treatment directly affecting CD24 and CD166 subpopulations within SNU-1, or if other subpopulations within the SNU-1, for example the CD24⁻CD166⁻ population have their phenotype changed by the BaP treatment too.

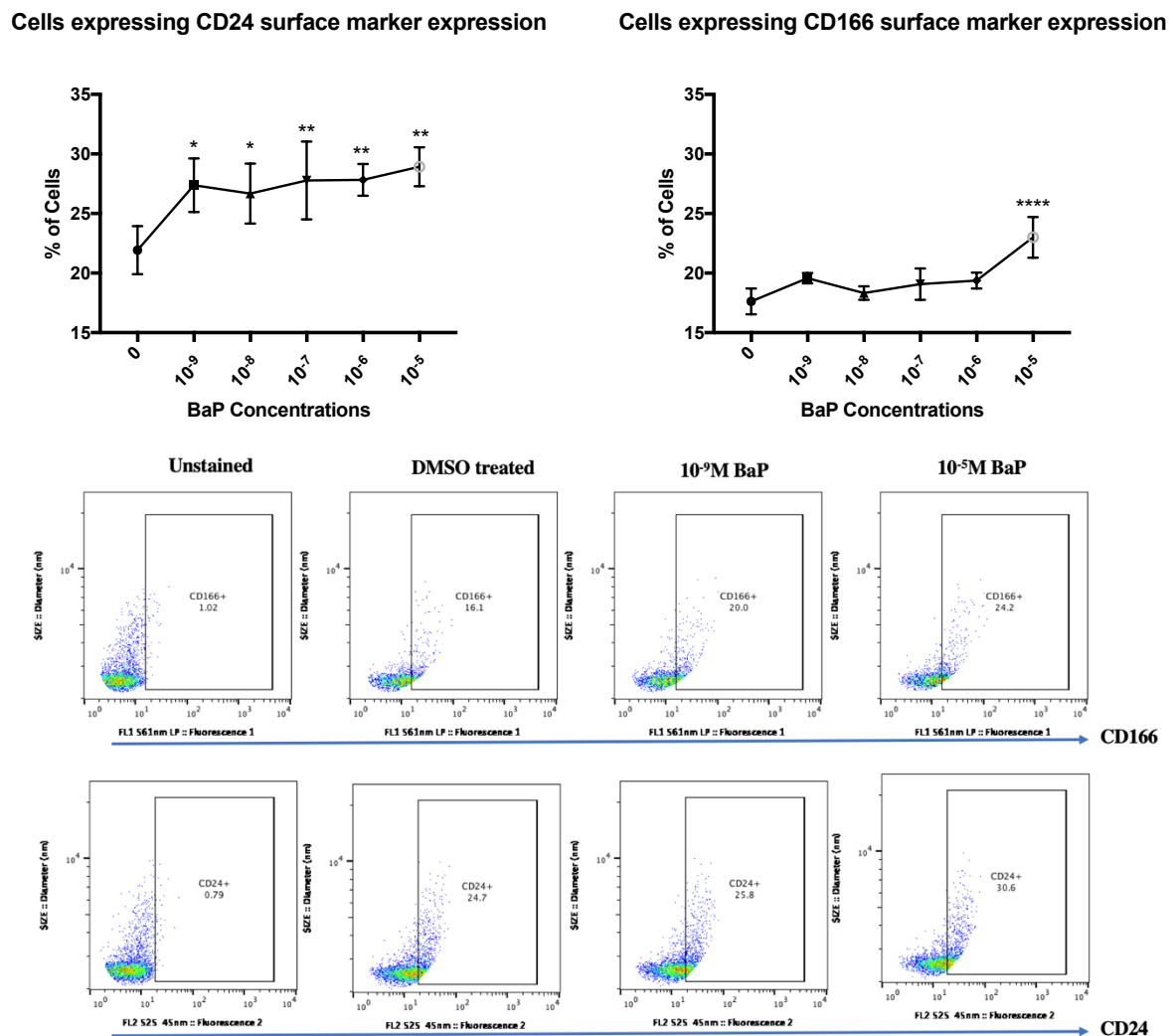


Figure 4.18: Invasion assay with SNU-1 population treated with BaP at 48 hours. Invading cells at the bottom well were stained and analysed with the flow cytometer. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed separately to investigate the effect of BaP concentration on the percentage of CD24 and CD166 at 48 hours.

4.4 Discussion

4.4.1 Genotoxicity effects caused by BaP treatment in SNU-1 cells

In this study, I found that BaP can induce phenotypic change to the SNU-1 cell line. BaP treatment also induced AhR expression and the expression of CYP1 family gene. A previous study in mice suggested that *AhR* regulation is central in modulating carcinogenesis [186]. The same study also found that AhR-deficient mice showed no response to BaP and consequently no obvious tumour growth at the end of 18-week treatment. In addition, BaP did not induce any *CYP1* expression in the skin and liver; in contrast to the enhanced expression of AhR-positive (+/+ and +/- genotype) bearing mice. Mice with these genotypes exhibited *CYP1* expression and also tumour growth [186]. Therefore, evaluating the expression of *CYP1* family genes is one of the crucial indicators of the molecular response to BaP induction as these genes encode the cytochrome P450 enzymes, which are involved as a Phase 1 drug metabolism of BaP [269]. Most importantly, the by-product of the metabolism of BaP yield B[a]p-7,8-dihydrodiol-9,10-epoxide (BPDE) – can bind to DNA, cause pre-mutagenic adducts and form micronuclei [183], [184]. Inspecting micronuclei formation is a reliable method to measure the effect of genotoxicity [270]. BaP is a known genotoxin that induces micronuclei formation [271] in stomach by formation of DNA adducts as a mutagenic response [177]. Thus, the micronuclei formation observed in BaP treated SNU-1 cells is a result of the genotoxic effect caused by BaP (Refer to Figure 4.19 for the complete diagram of the proposed process). The covalent bonding of a carcinogenic by-product of BaP metabolism, BDPE, to guanine residues of DNA (such as adenine or guanine) causes DNA adduct formation (Figure 1.11). The mutagenic effect of this binding, if left unrepaired or mis-repaired, might eventually lead to gene mutation and carcinogenesis [177]. In this experiment, genetic damage was observed on the basis of micronuclei formation. Furthermore, Etoposide (an agent known to disrupt DNA as a positive control) caused the inhibition of topoisomerase II, which disrupts transcription and replication. The profound effects of this inhibition on cell physiology become apparent with the rapid formation of one or more pockets of genetic material of removed DNA strands in the cytoplasm that have not been incorporated into the main nucleus of a cell. This results in the phenomenon called a micronucleus formation¹³, which was investigated in Section 4.3.3 of this chapter. On the other hand, mutagens such as BDPE exert their clastogenic endpoints by inducing disruption, or breakage of chromosomes [271]. This event leads to a form of

¹³ Nitiss, J. L. (2009a). DNA topoisomerase II and its growing repertoire of biological functions. *Nature Reviews Cancer*, 9(5), 327–337; see also: Nitiss, J. L. (2009b). Targeting DNA topoisomerase II in cancer chemotherapy. *Nature Reviews Cancer*, 9(5), 338–350.

mutagenesis, whereby if a cell is not killed, or does not undergo apoptosis, it may cause genomic instability within the cell. This leads to an abnormal transformation during which the cell's replicative potential increases, which is the basis of cancer formation. It is important to note that 72 hours treatment period used in this experiment is insufficient to cause notable mutations, if any at all, as they may require prolonged accumulation of genetic damage in the treated cells. Nevertheless, the micronuclei formation observed in the experiment, in response to BaP treatment, activates *AhR* to trigger CYP genes activation (Figure 4.2). The activation of CYP genes will produce the necessary enzymes required to metabolise the BaP, which can potentially yield various harmful by-products, especially BDPE, whose clastogenic effect was sufficiently shown in Section 4.3.3 by the increased frequency of micronuclei found in the treated cells.

4.4.2 Did AhR response trigger stemness expression and EMT in SNU-1 cells?

Recently, Sherr and colleagues discovered that when AhR is hyper-activated in breast cancer cell lines with environmental ligands, significant inductions of the expression of CYP family gene *IA1* and *IB1* were observed, which was associated with the increased expression of stemness expression and markers and with invasion/migration related genes [239]. This discovery is in line with my findings. BaP acted as an AhR-ligand induce the expression of the CYP family genes *via AhR gene* activation, and the induction effect mirrored the stemness genes' expression pattern in *OCT3/4*, *SOX2*, and *NANOG* within 24 hours (Figure 4.6). In addition, the expression patterns appeared to be dose dependent. It is possible that by increasing bioaccumulation of AhR-ligand, binding potential to AhR was increased with the induction of the xenobiotic response element in the promoter upstream region of CYPs genes and other genes [175], [272], [273]. My findings are supportive of the hypothesis that the induction of *AhR* with an exogenous ligand such as BaP may upregulate multiple stem cell associated genes. In fact, many genes induced by BaP in this study (such as *CYP1B1*, *SOX2*, *OCT3/4*, *NANOG*, and *Snail*), which are involved in human stem cells and migration/invasion-associated gene promoters, have consensus sequences¹⁴ with *AhR* response elements in Transcriptional Regulatory Element Database [239], suggesting that they may be directly regulated by *AhR*.

¹⁴ "A consensus sequence consists of the most commonly encountered bases at each position in a group of related sequences", Chapter 10, page 250 [274].

Metabolic activation of BaP that leads to DNA damage

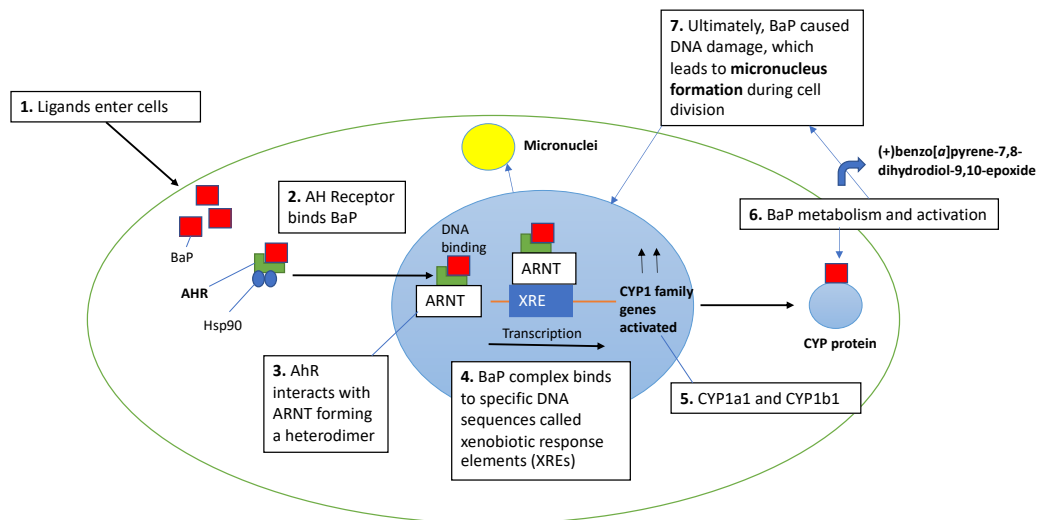


Figure 4.19: Benzo[a]pyrene (BaP) is one of the best studied polycyclic aromatic hydrocarbons (PAH) classified as group 1 human carcinogen by the International Agency for Research on Cancer (IARC) [176]. Classified as group 1 means that it is a confirmed human carcinogen [174]. When subjected to Benzo[a]pyrene (BAP) [177] or 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin, TCDD) [178], the ligand (e.g. BaP/TCDD) binds to Aryl hydrocarbon Receptor (AhR) and forms an AhR receptor complex (AhR + ligands). This complex translocates into nucleus and forms a heterodimer with Aryl hydrocarbon Nuclear Translocator (ARNT) [179], [180] to recognize a XRE binding site (Xenobiotic response element) [181], located at the upstream regulatory region of gene targets such as cytochrome P450 (CYP1A1 and CYP1B1 genes) [180], [182]. BaP metabolic byproduct by CYPs and epoxide hydrolase resulted in mutagenic and carcinogenic metabolites of B[a]p-7,8-dihydrodiol-9,10-epoxide (BPDE). BPDE can covalently bind DNA guanine residues to form pre-mutagenic adducts [183], [184]

4.4.3 AhR and CTNNB1: A possible link between stemness and EMT characteristics in SNU-1

There is an indication that AhR can act as a regulator of the Wnt- β -catenin pathway [188]. This canonical pathway is crucial for regulating stem cell pluripotency and its maintenance [275], [276], as well as for cancer signalling [275]. Notably, the evidence suggests that for a cell to acquire EMT characteristics, acquiring stemness characteristics would be the cell's first logical step, as it would allow cells to undergo phenotypic change, by regulating an embryonic pathway such as Wnt pathway [92]. *β -catenin*, coded from the CTNNB1 gene (a key molecule in Wnt/Wingless signal transduction and a key player in EMT), was detected in all sorted populations in Chapter 3 (Figure 3.7H, 3.7I, 3.7J).

Notably, it was highly expressed in CD166⁺ population (Figure 3.6H). In this study, the increased expression of *CTNNB1* was detected at higher dosages of BaP (Figure 4.12). Therefore, it is likely that AhR activation by BaP may induce CD26 and CD166 motility potential via *CTNNB1* upregulation (Figure 4.18) at 48 hours. It was suggested that EMT and stemness are both features of CSCs; in some tumours, both processes are believed to be parallel events [150]. In ovarian [277] and breast cells [278], *Snail* over-expression induces stemness cell invasion and metastasis. The possibility that BaP exposure inducing plasticity in the SNU-1 resulted in phenotypic change and cell transformation towards more aggressive “mesenchymal phenotype” due to the activation of *Snail* in SNU-1 cannot be dismissed. BaP exposure also induced important toxicogenomic outcomes which are associated with cellular transformation processes, typically seen during carcinogenesis transformation [269].

4.5 Conclusion

To summarise, AhR transcriptional targets such as *CYP1A1* and *CYP1B1* are possibly induced by *AhR* and Wnt/ β -catenin cooperation [279]. Wnt signalling (*CTNNB1*) crosstalk with the expression of stemness factors such as SOX2, OCT3/3 and NANOG is probable [280]. Although SNU-1 lacks protein expression of *E-cadherin*, the use of demethylating agents such as BaP can restore the *E-cadherin* expression [31], [281] due to the methylation at the promoter region [22]. Based on my findings in this chapter, I suspect BaP-mediation can trigger EMT through *Snail* repression and *AhR* activation and Wnt-signalling pathway which caused the migratory enhancement of SNU-1. With BaP treatment, the plasticity of CD24⁺ in unsorted SNU-1, showed slight advantage over CD166⁺ in acquiring invasive phenotype. Based on the findings in this chapter, the full understanding of the cells' molecular response to induced plasticity may be connected with the subpopulations that the cells belong to. Thus, to effectively understand the extent of phenotypic change caused by genotoxicity of BaP to the sub-populations, the next logical step would be to introduce the BaP treatment to the sorted subpopulations of CD24 and CD166.

5 BaP Induced Plasticity of Sub-populations of SNU-1 Gastric Cancer Cell Line

5.1 Introduction

BaP is an established ligand of the aryl hydrocarbon receptor (*AhR*) [192]. When a cell is exposed to Benzo[a]pyrene (BaP), *AhR* binds to this ligand to form an *AhR*-BaP complex [186]. The *AhR* – receptor complex can diffuse into the nucleus, dimerize with Aryl hydrocarbon Nuclear Translocator (*ARNT*) [181] and bind to xenobiotic response element (XRE) in the promoter region of gene targets such as *CYP1A1* and *CYP1B1* to initiate transcription [236]. The translation of these genes produces enzymes that will metabolize BaP. One of the metabolic products resulting from CYP enzyme metabolism of BaP is B[a]p-7,8-dihydrodiol-9,10-epoxide (BPDE), which can covalently bind DNA guanine residues to form pre-mutagenic adducts [282], which, if left unrepaired, may lead to genetic mutation that can impair gene function (Refer to Figure 4.19).

Although in theory BaP's primary mode of action is by causing genotoxicity, the nature and extent of molecular responses at the gene and protein level varies [283]-[285]. For example, broad similarities were found in gene expression profiles between lung and liver following BaP exposure: perturbed pathways involved in xenobiotic metabolism, *AhR* signalling and DNA damage response were observed [286]. Others have reported that although the mutation frequencies found in these two tissues are quite similar after BaP treatment, the level of formation of DNA adducts was higher in lungs than in liver, and the transcriptional response (fold change and total number of genes) was greater in lungs as well [283]. Moreover, a study of genotoxic screening of 24 compounds (including BaP) in 4 different human cell lines (HepG2, Hep3B [hepatoblastoma cells], and ACHN [renal adenocarcinoma], LS-174T [epithelial colorectal adenocarcinoma cells]) found distinct biotransformation properties between these various cell lines [285]. Therefore, it is possible that the possible outcomes of BaP induction of phenotypic change may vary between the subpopulations of SNU-1 too.

Others have reported that as well as its ability to damage DNA, BaP can induce a number of other cellular responses that contribute to carcinogenicity [189], [283], [287]. For example, a study on gastric cancer (GC) cell lines (SGC-7901 and MNK-45) found that BaP promoted proliferation and increased migratory and invasive capabilities through upregulation of MMP9 and *C-MYC* expression [288]. In

the previous chapter, I showed that BaP can induce migration and invasion of the SNU-1 cells. In order to achieve this, various EMT-inducers and key genes in this process such as *Snail*, *MYC*, and *CTNNB1* were induced. The induction effects of these genes are widely known to encourage stemness in cells and are associated with increased migration and invasiveness [166], [196], [278], [289], [290]. In addition, I also found that the stemness gene expressions such as *SOX2*, *OCT3/4* and *NANOG* were also induced with BaP treatment.

Thus, I suspect that the carcinogen BaP activation and DNA damaging process to elicits a range of biological responses are possibly cell or population-dependent. In chapter three, I described two subpopulations (CD24⁺ and CD166⁺) in the SNU-1 gastric cell population, which are phenotypically different from each other, in addition to a population depleted of both subpopulations as a comparison. Thus, the question arises whether the phenotypically distinct subpopulations respond differently to BaP treatment?

5.1.1 Hypothesis

In this study, I intend to explore the extent of the plasticity potential of the subpopulations when treated with BaP to address the hypothesis that:

- *AhR* activation may cause sorted CD24 and CD166 subpopulations to undergo phenotypic change to increase invasiveness and migratory capacity, CSC marker expressions, and stemness factors.
- Within the SNU-1 GC cell line, there are plastic subpopulations that can undergo phenotypic change and also acquire stem-like phenotypes with BaP treatment.

5.2 Materials and Methods

5.2.1 Cell culture

Human gastric cancer cell line SNU-1 was purchased from American Type Culture Collection (ATCC) and cultured as recommended by ATCC. SNU-1 was routinely cultured in ATCC RPMI-1640 Medium (ATCC® 30-2001™) supplemented with 10% foetal bovine serum (FBS), 100units/ml penicillin, 100µg/ml streptomycin (GIBCO, Life technologies) in T75 flasks. Cells were incubated at 37 °C in a humidified incubator (5% CO₂).

5.2.1.1 Cell treatments

Cells were seeded at a density of 1×10^5 cells per well in a 24 well plate. For treatment with dietary carcinogen, cells were treated with BaP (0- 10 µM, Sigma-Aldrich). The BaP chemical was dissolved in DMSO (Dimethyl sulfoxide, Sigma-Aldrich), and a negative control (treatment without adding any BaP; annotated as “0” in the graphs), made of 0.2 % DMSO in distilled H₂O, was used in all treatments. Early passage cells were used for all experiments (passage 7-10).

5.2.2 Sample preparation for magnetic sorting of SNU-1 subpopulations

CD166 microbead, purchased from Miltenyi Biotec, contains 1 ml CD166-biotin, human (130-106-574, monoclonal CD24) antibody conjugated to biotin (clone: REA442; isotype: recombinant human IgG1), and 2 ml of Anti-Biotin Microbeads (130-090-485, MicroBeads conjugated to monoclonal anti-biotin antibodies; isotype: mouse IgG1). CD24 microbead kit (130-095-951) was also purchased from Miltenyi Biotec. The kit comprised of 1 ml CD24-Biotin, human (monoclonal CD24) antibody conjugated to biotin (clone: 32D12; isotype: mouse IgG1), and 2 ml Anti-Biotin MicroBeads (MicroBeads conjugated to monoclonal anti-biotin antibodies; isotype: mouse IgG1). A buffer solution containing phosphate-buffered saline (PBS), pH7.2, was prepared with the addition of 0.5% bovine serum albumin (BSA) for use in the sorting process. This solution was used throughout the magnetic separation process. LS columns (130-041-306, Miltenyi Biotec) were used for positive selection of cells strongly expressing the CD24 antigen. Cell lysate obtained from cell culture was washed once with PBS and centrifuged at 300×g for 10 minutes. The cell pellet was resuspended in the sorting buffer made earlier, and cells were passed through moistened 30 µm nylon mesh (Pre-Separation

Filters (30 μm), 130-041-407, Miltenyi Biotec) to remove any cell clumps which could clog the column during the sorting process. Once cell number had been determined, minimum 1×10^7 cells were used for the magnetic sorting. The suspension cells were centrifuged at $300\times g$ for 10 minutes, and the supernatant was aspirated completely. The cell pellet was resuspended in 40 μL of the buffer, and 10 μL of CD24-Biotin. CD166-biotin was added (for 10^7 total cells). Staining antibodies (CD24-FITC, CD166-PE, refer to Chapter 2 for details) was added as well to the mix for flow cytometry analysis at the end of magnetic separation. The solution was mixed well and incubated for 15 minutes in the dark in the refrigerator at 2–8 $^{\circ}\text{C}$. Higher temperature or more prolonged incubation may promote non-specific cell labelling. Later, the cells were washed with 2 ml of the sorting buffer and centrifuged for $300\times g$ for 10 minutes, and the supernatant was removed entirely. The cells were then resuspended in 500 μL of the buffer, and magnetic separation process begins.

5.2.2.1 Magnetic separation of subpopulations CD24 and CD166

LS column was placed in the magnetic field of the MACS separator (Refer Figure 3.1). The column was activated by being rinsed with 3 ml of the sorting buffer made earlier. Slowly, the cell suspension was added onto the column, and flow through was collected. The flow-through will contain unlabelled cells. To maximise the efficiency collection of unlabelled cells, the column was washed with 3 ml sorting buffer three times. The LS column was removed from the separator and placed on a new collection. 5 ml buffer was pipetted onto the LS column, and immediately, cells were flushed out from the LS column by using the plunger supplied with the column. This step fulfilled the first enrichment process. The labelled fraction of cells in the collection tube was analysed for CD24⁺/CD166⁺ percentage with a flow cytometer, Moxi Go II (Orflo, Moxi Go OS v4.3). For the second enrichment process, and to increase the purity of the labelled fraction of cells, the eluted labelled cell fraction was passed over the second LS column, and separation procedures described above were repeated. The eluted unlabelled cell fraction was also passed over to another second LS column, following the same separation procedure. Once the elution process finished, the LS columns was placed onto a new collection tube and the remaining labelled cells in the magnetic column were flushed out.

5.2.3 Flow cytometry analysis

Labelled fraction and unlabelled fraction of cells from magnetic separation were processed for flow cytometry analysis. Firstly, cell counts were performed using an automated cell counter machine (Moxi Z from ORFLO Technologies), and 1×10^6 cells were added into each FACS tube and incubated on ice for 15 minutes. New medium (1 ml) was added to as many tubes as required and centrifuged for $500 \times g$ for 5 minutes. PBA (30 μ l of 1% Bovine serum albumin dissolved in Phosphate buffered solution) containing 3 μ l of FcR Blocking reagents (Miltenyi Biotec) was added to each tube containing the cells to reduce non-specific binding of the antibodies. PBA also stabilizes the cell membrane and prevents antibodies from sinking into the membrane.

Next, supernatants were discarded, and 10 μ l of an antibody of each CD markers (refer to Table 5.1 for concentrations) diluted in 100 μ l of staining buffer were added to 1×10^6 cells' pellet in each respective tube labelled with the corresponding CD marker being used. Then the tubes were incubated for 30 minutes on ice. It is worth noting that antibodies were titrated to determine optimal antibody concentration for live-cell labelling to achieve the best discrimination between positive and negative cells, while keeping nonspecific antibody binding to a minimum, according to the protocol's recommendation [210]. Finally, 500 μ l PBA was added to each tube, and the samples were centrifuged for $400 \times g$ for 15 minutes, and the supernatant was discarded to remove excess antibodies. Cell pellets were resuspended in 1 ml of PBA and were examined with flow machine (BD FACS Fortessa). The results were analysed using FlowJo software, version 10.1r1, on Mac OS X El-Capitan Operating System, version 10.11.5.

Table 5.1 CD markers used in the experiments:

CD Markers	Supplier	Supplier items code	Concentration (per 10 μ l)
FITC Mouse Anti-Human CD24	Becton Dickinson	555427	0.2ug/ μ l
PE Mouse Anti-Human CD166	Becton Dickinson	559263	0.0125ug/ μ l

Fluorescence Minus One Control or FMO control are used in this experiment. The FMO control is used to correctly interpret flow cytometry data, and especially in the case where the percentage population of interest is meagre; it acts as reasonable quality control. It is used to identify and gate cells in the context of data spread due to the multiple fluorochromes in each panel. An FMO control contains all the fluorochromes in a panel, except for the one that is being measured. Therefore, since there were only two antibodies used, the FMO for each sample would be the unstained sample without any antibody.

Table 5.2 Experimental design with FMO for SNU-1 cells.

Antigen	FITC	PE
CD24 FMO	-	CD166
CD166 FMO	CD24	-

5.2.4 Gene Expression Studies

5.2.4.1 RNA extraction

RNA was extracted from at least 1×10^6 cells using the RNeasy kit (Qiagen). The kit is supplied with Qiagen's proprietary RLT lysis buffer, RW1 RNA precipitation buffer, and RPE RNA washing buffer. Harvested cells were centrifuged at $1500 \times g$ for 5 minutes to pellet the cell. RLT buffer plus (add 10 μ l β -mercaptoethanol (B-ME) to 1ml RLT buffer) was added to pelleted cells (350 μ l for $< 5 \times 10^6$ cells / 600 μ l for $< 1 \times 10^7$ cells), and vortexed for 30 seconds. The lysate was centrifuged for 3 minutes at maximum speed ($18000 \times g$). The supernatant was removed by careful pipetting, and the lysate was transferred to the genomic DNA Eliminator spin column that came with the RNeasy kit and placed in a 2 ml collection tube to remove DNA contaminations. Columns were centrifuged for 30 seconds at $8000 \times g$. Columns were discarded and flow through was kept aside. RNA precipitation was done by adding 1 volume of 70% ethanol (same volume as RLT volume amount used, *e.g.* 350 μ l/600 μ l). Next, up to 700 μ l of samples transferred to RNeasy spin column placed in a 2 ml collection tube, and centrifuged for 15 seconds at $8000 \times g$, and flow through was discarded. RNA in the RNeasy spin column was washed with 700 μ l of RW1 buffer, followed by washing with 500 μ l of RPE buffer; both times centrifuged at $8000 \times g$ for 15 seconds. Precipitated RNA was washed for the final time with 500 μ l RPE buffer, centrifuged for 2

minutes at 8000×g. For RNA collection, RNeasy spin columns were placed in a new 1.5 ml collection tube, and 30 µl of RNAase-free water was added to elute the RNA into collection tubes. Samples were centrifuged for 1 minute at 8000×g. Optionally, the column may be spun again with another volume of water or the RNA elute to maximise RNA collection. Finally, RNA extracts in each tube were labelled accordingly and quantified by UV spectroscopy (UV-VIS Nano-spectrophotometer, Implen, Essex, UK) with purity assessed from 260/280nm and 260/230nm ratios. Extracts were stored at -80°C until used.

5.2.4.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

cDNA strands were synthesized by using a reverse transcriptase kit, Superscript II enzyme (ThermoFisher). RNA extracts (100-500 ng) from each sample were added to 300 ng of random primers, heated for 5 min at 65°C and then quickly placed on ice. Each tube (containing the RNA extracts and random primers) was incubated with 0.5 mM dNTPs, 1x first strand buffer, 8µM dithiothreitol and 100 units of Superscript II reverse transcriptase (Invitrogen, Life technologies) for 10 min at 25°C, 90 min at 42°C and 15 min at 70°C in a thermocycler (Peltier Thermal Cycler PTC-200, MJ Research, Waltham, Massachusetts, USA). An mRNA reverse transcription kit was used for mRNA expression according to the manufacturer's protocol (Taqman, Applied Biosystems, Life Technologies). Quantitative-Polymerase Chain Reaction (qPCR) was performed using Taqman probes pre-designed gene expression assays (detailed in Appendix A) and FAST PCR master mix (TaqMan, Applied Biosystems, Life Technologies), and measured in a StepOnePlus fast real-time PCR system (Applied Biosystems, Life Technologies) according to the manufacturer's protocol. GAPDH was used to normalise cellular mRNA expression, respectively (Appendix A). Gene expression was quantified using the delta-CT method.

5.2.5 Invasion and Migration Assay

5.2.5.1 Invasion assay

Matrigel invasion chambers were obtained from Corning (USA, catalogue number: 354480). The cell culture inserts containing 8-micron pore size PET membraned had been pre-treated with Matrigel Matrix by the manufacturer and could be placed into a standard 24-well plate. The chambers were placed in a 24-well plate, and 500 µl of warm RPMI culture medium were added to the interior of the chambers, and another 500 µl of the medium were added to the well to moist the exterior of the chambers. After 2-hour rehydration the medium was removed from the chambers and was ready to use for an invasion assay. To perform the invasion assay, hydrated chambers were placed on the 24-well plate, with 500 µl of RPMI culture medium supplemented with 1% dextran-coated charcoal-stripped FBS added in the Matrigel chambers, and 500 µl of culture medium with 10% FBS were added at the bottom well (where the chambers are placed) to act as a chemoattractant. 1×10^4 cells were plated into each chamber. Then, five concentrations (1×10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} M) of BaP were added into the chambers and left in cell incubator for 48 and 72 hours. After the incubation period, 1 ml of PrestoBlue was added to each bottom well and further incubated for 30 minutes respectively for each time point per manufacturer's recommendation. The fluorescence was recorded on a microplate reader (Synergy Hybrid, Biotek) at a wavelength of 535 nm (excitation) and 615 nm (emission). Top read and bottom read format were recorded, due to the different adherence nature of the cells.

5.2.5.2 Migration assay

For migration assay, 1×10^4 cells per well were plated in the upper chamber of a 96-transwell insert system with an 8 µm pores PET membrane (no Matrigel layer; BD Falcon, Oxford, UK) and with 500 µl of RPMI culture medium supplemented with 1% dextran-coated charcoal-stripped FBS. Meanwhile, 500 µl of culture medium with 10% FBS were added at the well (where the chambers are placed) to act as a chemoattractant. Similarly, to the invasion assay, five concentrations (1×10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-6} M) of BaP were added into the chambers and left in cell incubator for 48 and 72 hours. In both cases, for invasion and migration assay, the number of cells migrated into the well was counted with Moxi Z cell counter (Orflo, USA). Results are expressed as the number of cells migrated to the lower chamber.

5.2.6 Micronucleus assay

Micronucleus (MN) assay was performed according to OECD guidelines and based on the modified protocol established in our laboratory [259], [260]. 2×10^4 cells were seeded per well of a 24-well plate for 2D cell culture and grew in stripped FBS RPMI for 72 hours. Stripped FBS RPMI medium was removed from the 24 well plate, and the cells were washed with PBS. Then, the cells were treated with various concentrations of BaP (1×10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-6} M) and cultured for further 48 hours with 10% FBS prior to harvest. Etoposide (Sigma-Aldrich) was used as a positive control in all assays at a concentration of 125nM as it is a known inducer of MN formation. Cells were then harvested and resuspended in culture medium containing 2% pluronic (GIBCO, Life technologies). Cells were fixed with 100% methanol onto microscope slides at a density of 2×10^4 cells per slide and stained for 60 seconds with acridine orange (0.1mg/ml dissolved in PBS, Sigma-Aldrich). Frequency of MN was scored randomly in 1000 cells per sample and four biological replicates were performed per treatment.

5.2.7 Statistical Analysis

Data were obtained from measurements made in at least four biological replicates and presented as a mean value, $\bar{x} \pm$ Standard deviation (SD). Raw data were recorded and organized in Excel 2016 Microsoft Office program. The statistical analysis was conducted in GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA). The normal distribution of the data sets was checked using the Shapiro-Wilk normality test. Then significant differences ($p < 0.05$) were determined using one-way analysis of variance (*ANOVA*) followed by a Dunnett post-hoc test, or a linear trend analysis and two-way *ANOVA* followed by a Bonferroni post-hoc test. Pearson's correlation coefficient test was used for the correlation analysis.

5.3 Results

5.3.1 How does BaP treatment affect AHR expression on CD24⁺/CD166⁺/ CD24⁻CD166⁻?

With time in culture during the BaP treatment, the sorted populations showed varied response (Figure 5.1). For example, CD24⁺ population showed no change in the mRNA level of BaP-associated genes with all doses of BaP treatment in the first 48 hours. However, by 72 hours, at higher BaP concentrations (10^{-7} and 10^{-5} M) *AhR* and *CYP1A1* genes were upregulated.

In contrast, CD166⁺ population showed more rapid phenotypic response to BaP treatment, especially by the 24th and 48th hours of treatment. At 24 hours, the concentration of BaP at 10^{-9} and 10^{-7} M triggers *AhR* and *CYP1B1* mRNA upregulation. However, at 48 hours, neither *AhR* nor *CYP1B1* was induced by the treatment, whilst *CYP1A1* showed elevated expression at higher doses of BaP. Interestingly, *AhR* expression decreased with an increasing BaP dose concentration at 72 hours' time-point (Figure 5.1).

In CD24⁻CD166⁻ population, at 24 hours both *AhR* and *CYP1A1* showed increased expression with BaP treatment, although the change was not dose-dependent. Oddly, *CYP1B1* mRNA level showed a significant decrease with increasing BaP doses. At 48 hours, only one dose of BaP (10^{-9} M) induced mRNA expression of all three genes. At 72 hours, *CYP1A1* showed increased expression only at the highest level of BaP concentration, however the other genes were unaffected (Figure 5.1).

In summary, these populations behaved differently from one another in response to BaP treatment. The phenotypic response of each population was differentially time-dependent. The CD24⁺ and CD24⁻CD166⁻ populations' responses involved *AhR* and *CYP1A1* induction, whilst CD166⁺ population response appeared to involve *AhR* and *CYP1B1* induction. Since *AhR* involvement seemed to be common within the subpopulations, it was decided to investigate whether the expression of *AhR*-related genes such as *ARNT* and *AhRR* could inform these observed effects in the sorted populations.

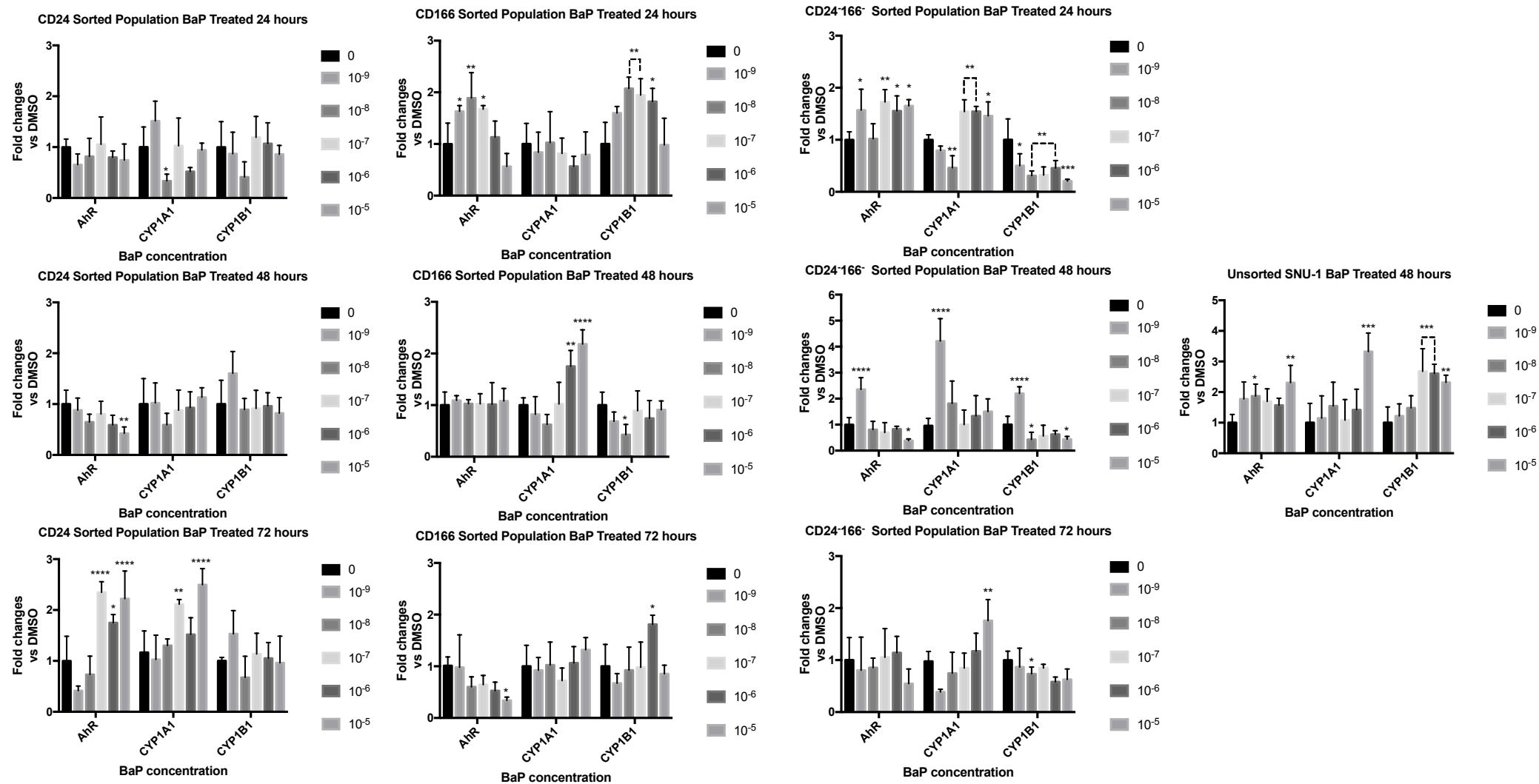


Figure 5.1: Sorted SNU-1 populations: CD24, CD16, and CD24-CD166-. The population's AhR, CYP1A1, and CYP1B1 genes' expressions when treated with BaP at various time-points. Statistically significant differences are shown for comparison between BaP treated and negative control samples (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Two-way ANOVA with Gene and BaP concentration as independent variables was performed separately on each sorted population to investigate the effect of BaP concentration on the expression of the respective genes (AhR, CYP1A1, and CYP1B1) at 24, 48 and 72 hours respectively.

5.3.1.1 How does BaP treatment affect AhR-related genes: ARNT & AhRR in different sorted populations of SNU-1?

In CD24⁺ population, AhR, AhRR and ARNT showed significant increase in expression when treated with BaP, particularly at 72 hours (Figure 5.2).

In CD166⁺ sorted population, AhR mRNA level increased at 10⁻⁹ and 10⁻⁷M BaP concentrations but not ARNT and AhRR gene expressions. At 72 hours, AhR gene expression decreased in a dose-dependent manner, whilst AhRR and ARNT expression level remained unchanged (Figure 5.2).

Interestingly, in response to BaP treatment, CD24⁻CD166⁻ population exhibited similar mRNA level and gene expression patterns for AhR, ARNT and AhRR across all time-points. This data shows that the different subpopulations respond differentially to the BaP treatment (Figure 5.2).

Consistent with this, it is important to note that in unsorted population of SNU-1, the effects of the BaP treatment on these same genes showed dose dependency. Taken altogether, it is possible that the heterogeneity in SNU-1 gastric cancer cell line population contributes significantly to the way cells respond to xenobiotic exposure such as BaP.

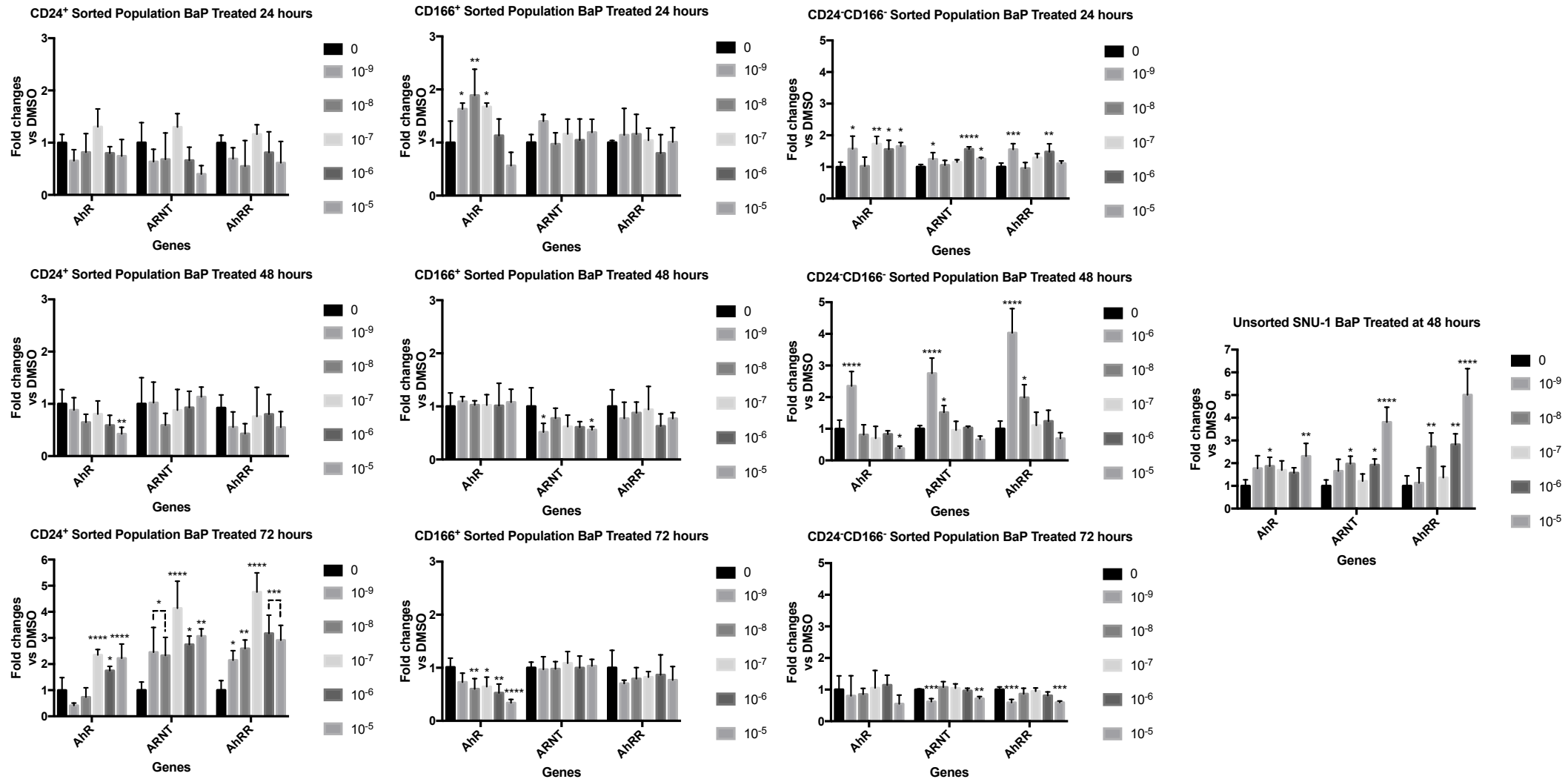


Figure 5.2: Sorted SNU-1 populations: CD24, CD16, and CD24⁺CD166⁻. The population's AhR related genes: ARNT and AhRR. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO) Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Two-way ANOVA with Gene and BaP concentration as independent variables was performed separately on each sorted population to investigate the effect of BaP concentration on the expression of the respective genes (AhR, ARNT, AhRR) at 24, 48 and 72 hours respectively.

5.3.1.2 Genotoxicity effect and cytotoxicity effect of various BaP dose to different SNU-1 populations

As previously discussed, metabolic activation of BaP produces genotoxic metabolites. Therefore, genotoxicity was assessed using a micronucleus formation assay. Using an optimised method, a dose-dependent increase in BaP induced MN frequency in sorted population of SNU-1 cell lines was observed. As shown in figure 5.3, interestingly, CD24⁺ sorted population exhibited a similar pattern of MN frequency to the unsorted population: increased MN frequency with increased dose of BaP. However, CD166⁺ sorted population showed the lowest overall MN frequency. Moreover, at the highest concentration of BaP, there was no difference in the MN frequency compared with the DMSO control. On the other hand, CD24⁻CD166⁻ population exhibited a modest BaP dose-dependent increase in MN frequency. Importantly, the positive control, etoposide, gave a strong positive MN response in all subpopulations. This is expected since etoposide induces MN through a completely different mechanism (topoisomerase II inhibition).

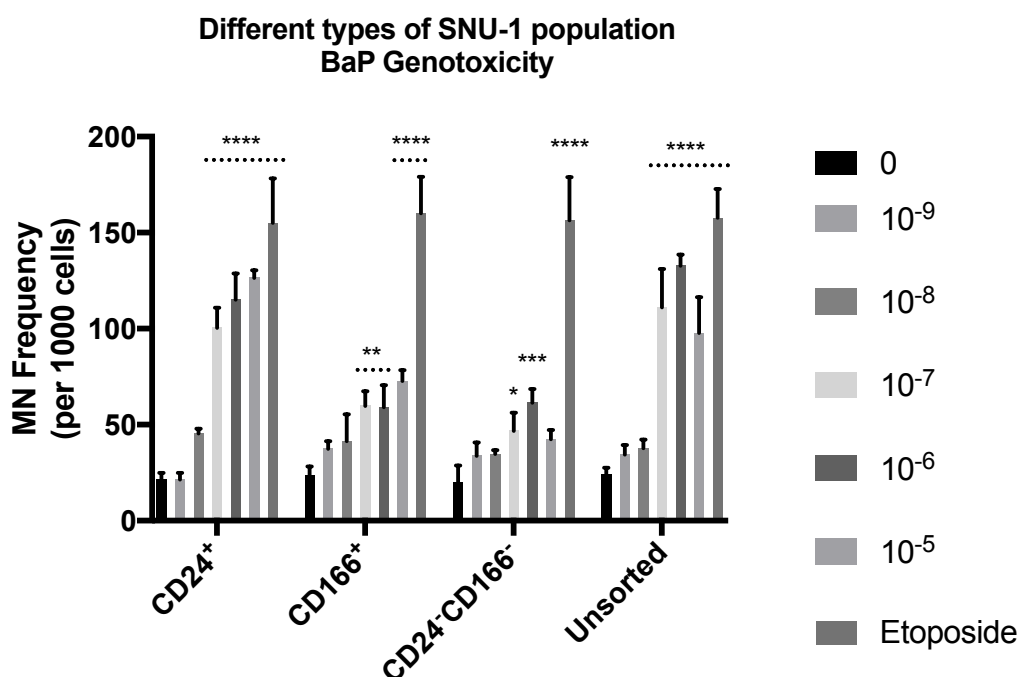


Figure 5.3: SNU-1 sorted and unsorted populations were grown and treated with various concentrations of BaP for 72 hours. The graph shows MN frequency per 1000 cells following the treatment. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). 2-way ANOVA with sorted population and BaP concentration as independent variables was performed to determine whether BaP concentration affects the frequency of the MN formation in each sorted population.

5.3.2 Does BaP treatment affect stemness (*SOX2*, *OCT3/4* and *NANOG*) phenotypic expression?

As shown in Figure 5.4, BaP treatment promotes changes to the phenotypic expression of the stemness genes, *SOX2*, *OCT3/4* and *NANOG*, with time and BaP concentration. In CD24⁺ population, an increased fold-change of mRNA level of the stemness genes was detected in a dose-dependent manner at 48 and 72 hours. A similar observation was found in CD166⁺ population, however, at 72 hours *SOX2* expression was not detected. In contrast, stemness phenotype in CD24-CD166- appeared to be more variable with a BaP dose at 48 and 72 hours. In unsorted population, BaP induced significant increase in expression of the stemness genes at higher dose concentrations at 48 hours. This finding showed that BaP seemed to affect stemness potential of SNU-1 sub-populations to varying degrees.

SOX2, OCT3/4 AND NANOG Expression in sorted population during 72 hours' time-point.

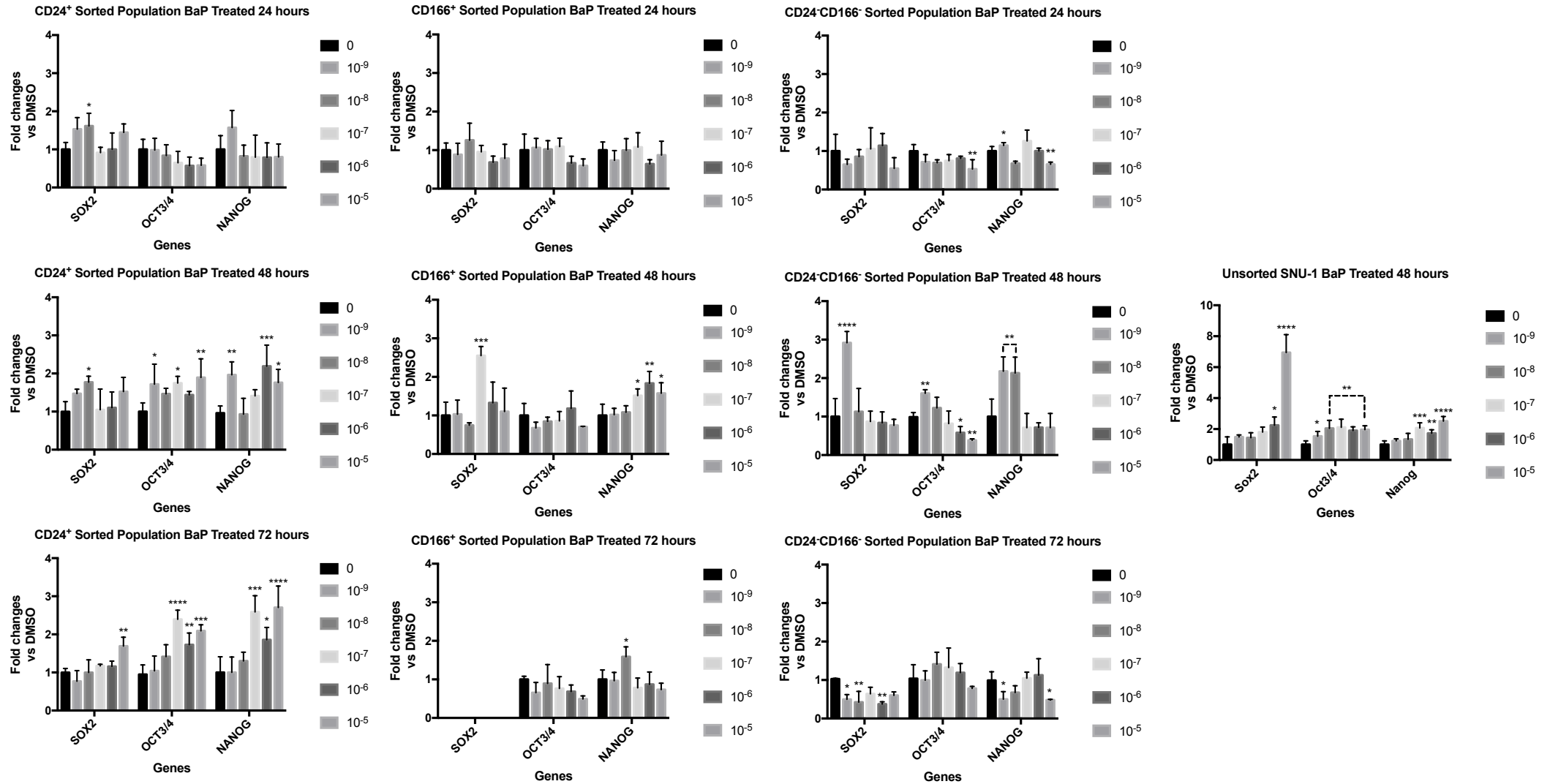


Figure 5.4: Sorted SNU-1 populations: CD24⁺, CD166⁺, and CD24⁺CD166⁻. The population's stemness genes expression: Sox2, Oct3/4, and Nanog. Statistically significant differences are shown for comparison between BaP treated and negative control samples (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Two-way ANOVA with Gene and BaP concentration as independent variables was performed separately on each sorted population to investigate the effect of BaP concentration on the expression of the stemness genes at 24, 48 and 72 hours respectively.

5.3.2.1 BaP effect on CD24 and CD166 gene expression in sorted population

Sorted populations were tested at three time points, 24, 48, and 72 hours, however, the effect of BaP treatment appeared to be optimal at 48 hours (Figure 5.5). The CD24 sub-population appeared to be more susceptible to phenotypic change with BaP treatment at higher doses ranging from 10^{-7} to 10^{-5} M, with the percentage of cells expressing CD24 increasing by 20% compared to the negative control (DMSO) (Figure 5.5). In contrast, CD166 population was more resistant and only showed CD166 upregulation at the highest dose of BaP (10^{-5} M). (Figure 5.5).

5.3.2.2 Does BaP affect the surface's protein expression of sorted sub-population of CD24 and CD166?

After BaP treatment, the P trend of CD24 and CD166 mRNA gene expression (Figure 5.5) mirrored the P trend of the surface protein marker expression (Figure 5.6) at 48 hours. Two- to six-fold change of mRNA level was detected in CD24 at 10^{-6} and 10^{-5} M BaP concentration, at 48 and 72 hours (Figure 5.6). Similarly at 48 hours, CD166 gene expression increased with BaP treatment compared to DMSO control (untreated); albeit the increase was lower than CD24's at 10^{-6} and 10^{-5} M. Consistent with my previous findings, the unsorted population of SNU-1 showed a significant increase in fold change of gene expression for both CD24 and CD166 at 48 hours after BaP treatment (Figure 5.7).

After treatment with BaP, CD24 gene expression was altered in CD24⁺CD166⁻ population at 24 and 72 hours. In the unsorted SNU-1, CD24 and CD166 gene expressions both showed significant increase in fold change with higher doses concentrations (Figure 5.7). In summary, CD24 and CD166 sub-populations both showed a phenotypic response with BaP, but it was more effective in a mixed population rather than in individual, sorted populations. These observations prompted questions about BaP treatment mediating phenotypic response in other genes.

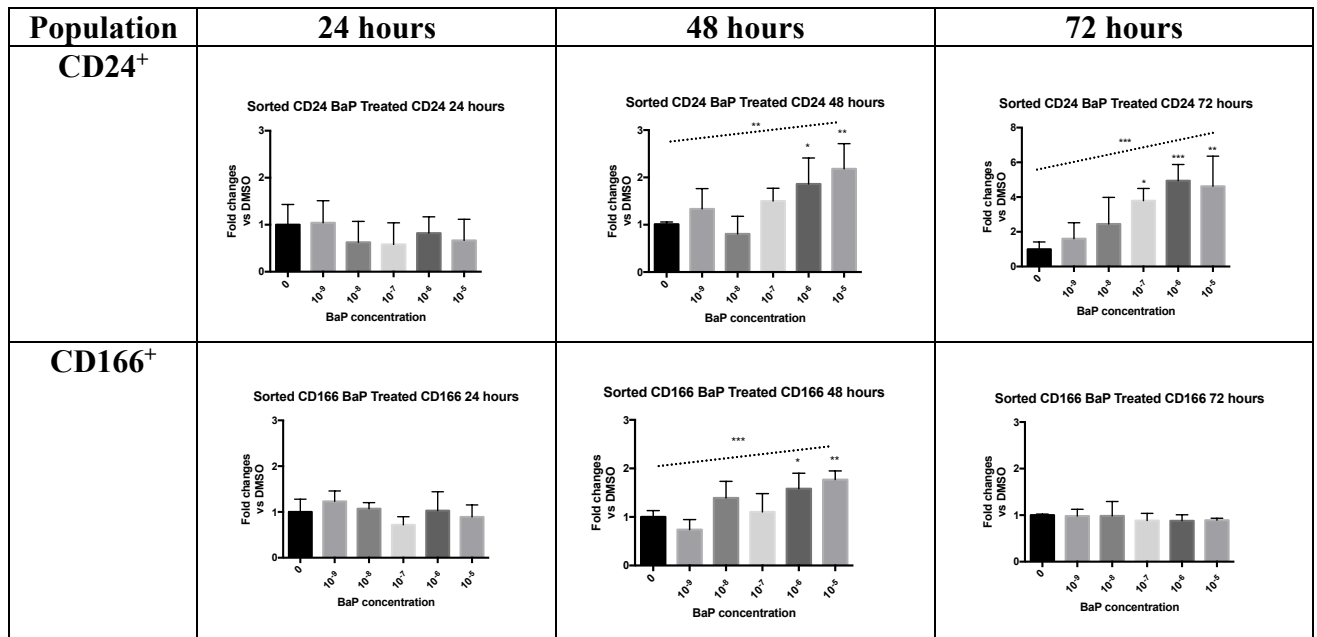


Figure 5.5: Difference in fold change in the mRNA level in the CD24⁺, CD166⁺ sorted populations (24, 48 and 72 hours). Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed separately to investigate the effect of BaP concentration on the mRNA level in the CD24⁺, CD166⁺ the sorted populations (24, 48 and 72 hours).

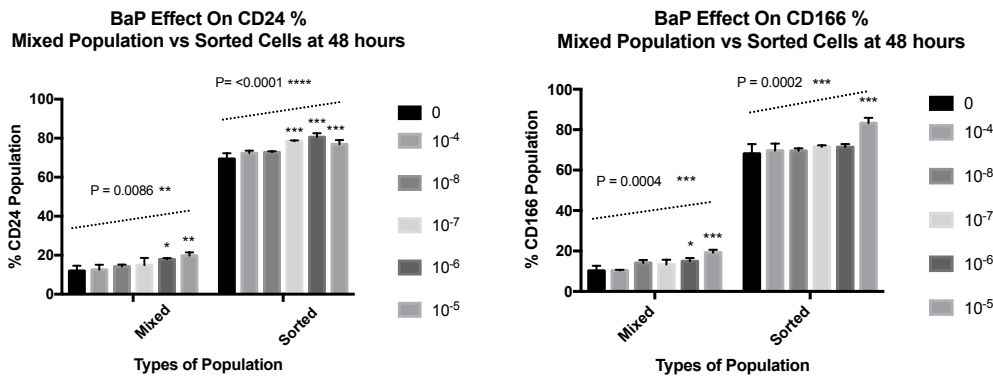


Figure 5.6: Comparison of CD markers' expressions on sorted and unsorted cells that were treated with BaP. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed separately to investigate the effect of BaP concentration on the percentage of CD24 and CD166 expressing population on the mixed and the sorted populations of CD24 and CD166 at 48 hours.

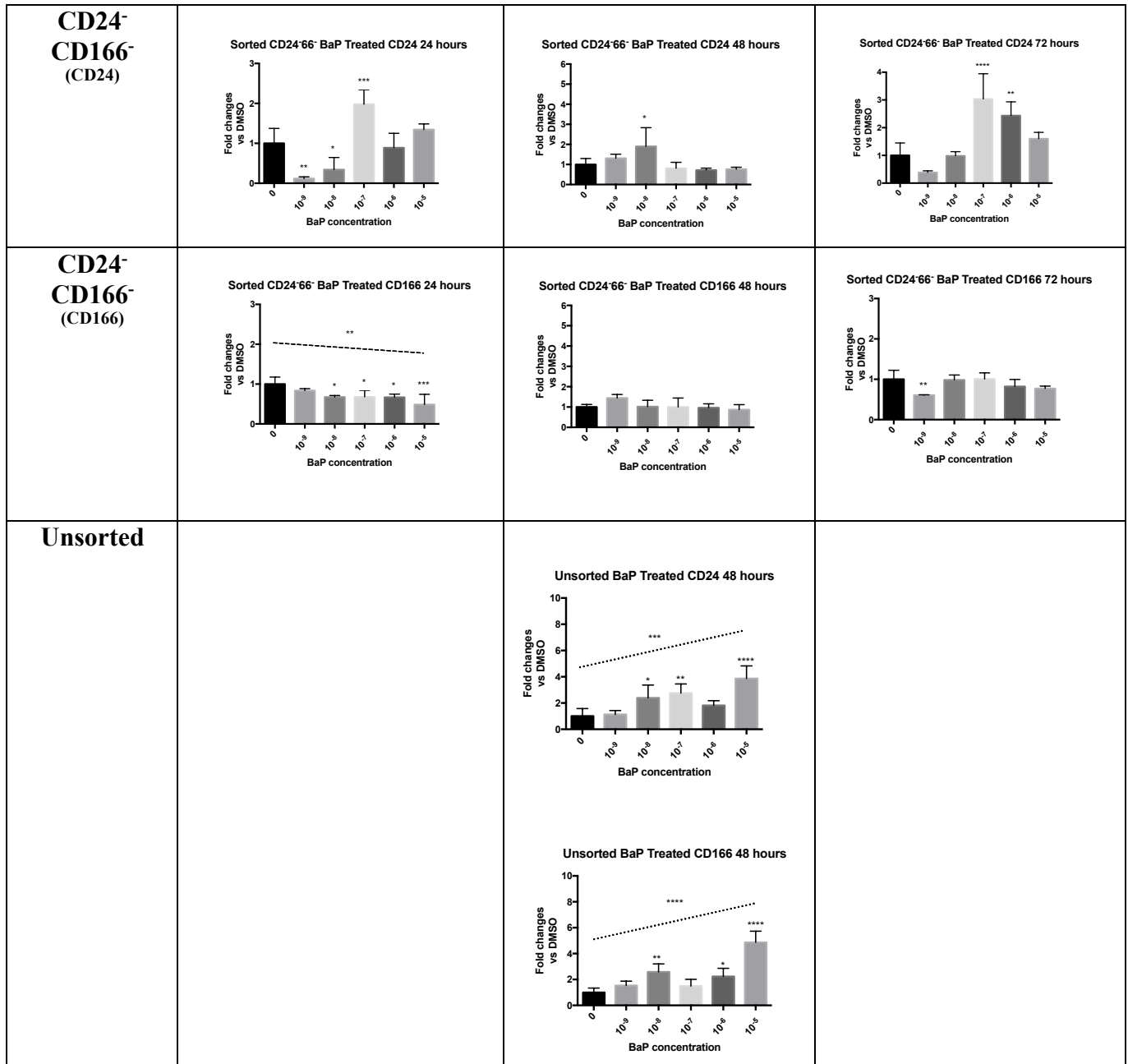


Figure 5.7: The fold change of CD24⁺CD166⁺ vs unsorted population. I have showed that 48 hours is the most optimal time-point for BaP induction effect for unsorted SNU-1 cell line. The sorted population in the absence of CD24 and CD166 populations showed a BaP effect of replenishing the population of interest. Statistically significant differences are shown for comparison between BaP treated and negative control (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed separately to investigate the effect of BaP concentration on the gene expression of CD24 and CD166 in the CD24⁺CD166⁺ sorted populations (24, 48 and 72 hours), in addition to BaP treated unsorted SNU-1 population at 48 hours.

5.3.3 EMT inducer genes: *SNAIL* and *TWIST1*, are expressed differently in SNU-1 sub-populations

SNAIL (*Snail*) gene is expressed differently in the sorted population (figure 5.8), for example in CD24⁺ population, its expression was induced at 72 hours, whereas CD166⁺ population only had a significant increase of mRNA level with the highest BaP concentration at 72 hours. On the other hand, CD24⁻CD166⁻ population showed a significant increase of *Snail* expression only at the lowest doses of BaP (10⁻⁹ and 10⁻⁸M) at 48 hours. The unsorted SNU-1 population showed a *Snail* mRNA induction profile similar to the one in CD24⁺ population (Figure 5.8). From this observation, it appears that 72 hours is the optimum time point for *Snail* gene activation with BaP.

Next, I examined another gene related to EMT: *TWIST1*, for all population types, sorted and unsorted at 72 hours. RNA was extracted from BaP-treated sorted populations of CD24⁺, CD166⁺ and CD24⁻CD166⁻ population and gene expression analysis for *SNAIL* and *TWIST1* were conducted (See Section 5.2.4.2). As previously mentioned, both *SNAIL* and *TWIST1* are key EMT-TFs. Based on the data (Figure 5.9), *TWIST1* gene expression was notably higher across all doses in the unsorted SNU-1, followed by the CD166⁺ sorted population, which exhibited an increased expression in a dose-dependent manner in response to BaP. However, in CD24⁺ sorted population, *TWIST1* gene expression was only expressed at the highest BaP concentration. This suggests that EMT-inducer genes, *Snail* and *TWIST1*, may be independent EMT-activating factors in CD24⁺ and CD166⁺ populations respectively.

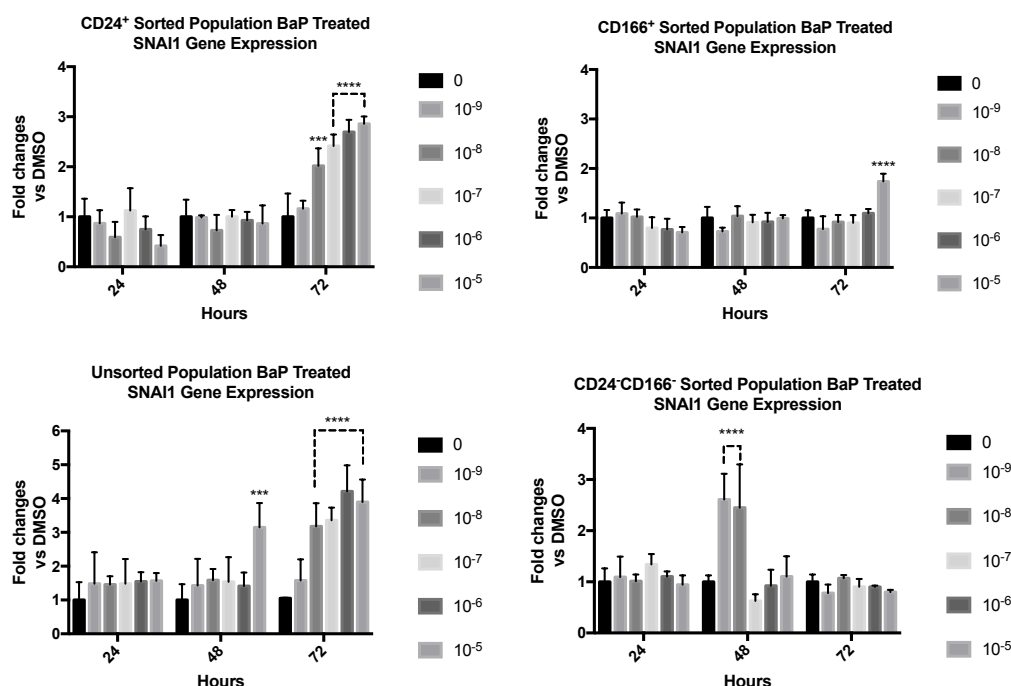


Figure 5.8: *Snail* expression on sorted populations, CD24, CD16, and CD24⁻CD166⁻, vs unsorted SNU-1 population. Statistically significant differences are shown for comparison between BaP treated and negative control samples (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Two-way Repeated Measures ANOVA with Time and BaP concentration as independent variables was performed to check whether BaP concentration has an effect on the *SNAIL* gene expression in the CD24 and CD166 sorted population during 72 hours' treatment.

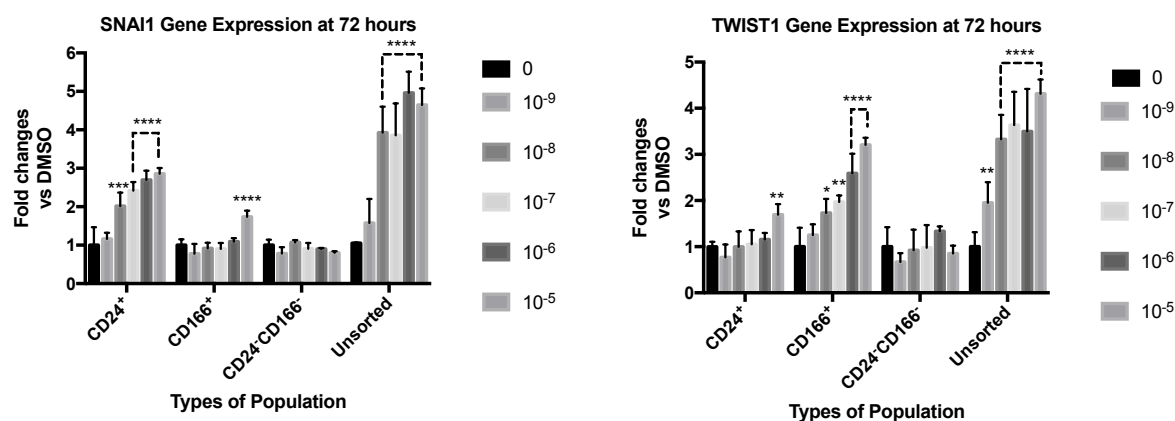


Figure 5.9: *Snail* and *Twist1* gene expressions in different types of SNU-1 populations, when treated with various doses of BaP at 72 hours. Statistically significant differences are shown for comparison between BaP treated and negative control samples (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Two-way Repeated Measures ANOVA with Type of Populations and BaP concentration as independent variables was performed to check whether BaP concentration has an effect on *SNAIL1* or *TWIST1* gene expression respectively at 72 hours.

5.3.3.1 Sorted SNU-1 populations migratory and invasion abilities are enhanced with BaP treatment

Previously in Chapter 4 (Figure 4.3), I hypothesized that *AhR* activation would also lead to SNU-1 phenotypic change to increase invasiveness and migratory capacity, CSC marker expressions, and stemness factors (see page 86). Then I evaluated the migratory and invasion abilities of unsorted SNU-1 when exposed to BaP

In this chapter, I hypothesized that *AhR* activation may cause sorted CD24 and CD166 subpopulations to undergo phenotypic change to increase invasiveness and migratory capacity, CSC marker expressions, and stemness factors. Interestingly, I found that more cells expressing CD166 surface marker invaded through the Matrigel than cells expressing CD24. However, migratory abilities of the sorted subpopulations of SNU-1 were unknown. Therefore, I designed a similar Transwell invasion assay to investigate this. The migrating/invading cells are estimated by measuring the fluorescent intensity using PrestoBlue Assay™. The effect of BaP on the sorted population was confirmed with Transwell migration and invasion assay, during which the cells exhibited increased motility with $\geq 10^{-7}$ M dose of BaP (Figure 5.10). CD24⁺, CD166⁺ and the SNU-1 unsorted population showed significant response to BaP treatment in a dose-dependent manner. Interestingly, CD24⁺CD166⁻ subpopulation, which lacks notable expression of *Snail* and *Twist1* and is not induced with BaP treatment, showed the lowest invasion and migratory response. In Figure 5.11, the cropped images of the Matrigel captured by microscope revealed that at the highest dose of BaP (10^{-5} M) show that cells in each population of SNU-1 gastric cancer cell line were indeed actively invading the Matrigel membrane, albeit to a lesser

extent in the CD24⁺CD166⁻ subpopulation. Thus, it is shown that BaP is able to induce phenotypic change to the subpopulations and endows them with enhanced migratory and invasive capabilities.

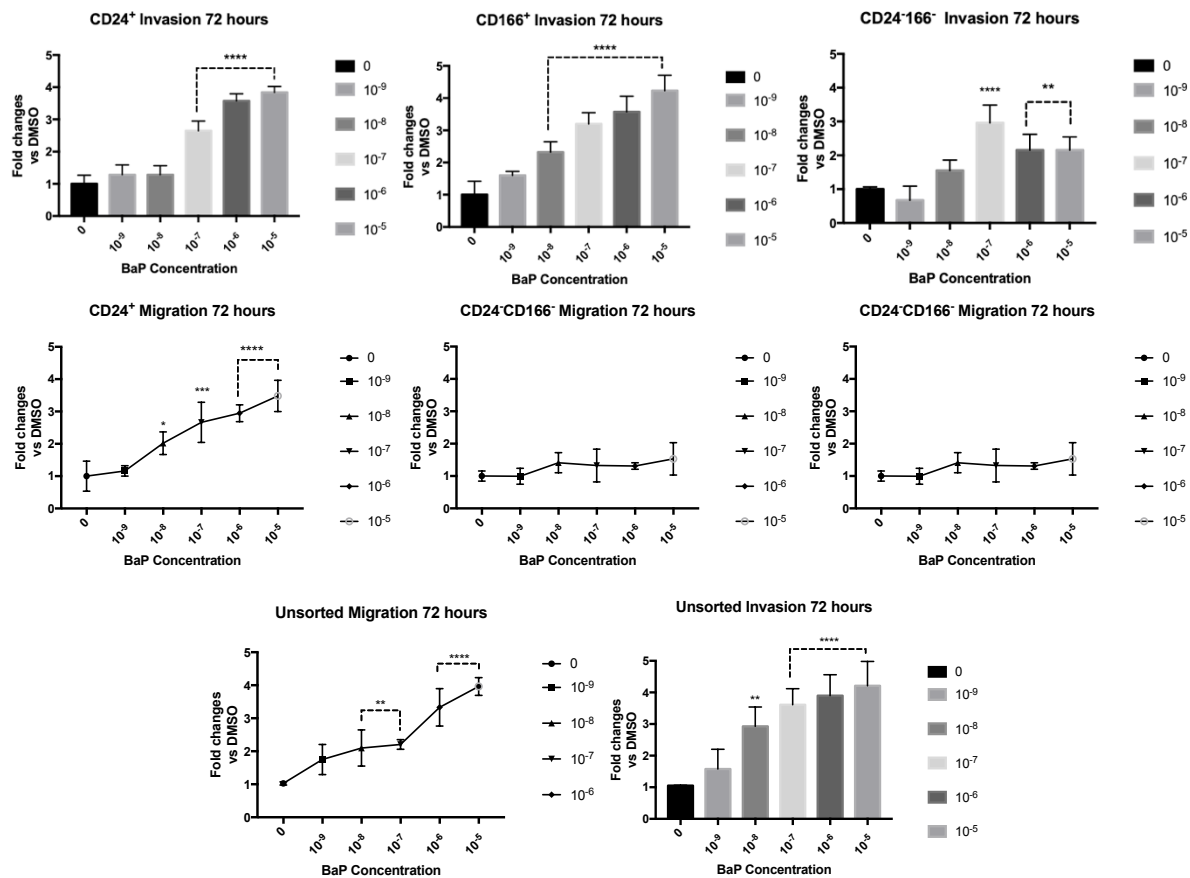


Figure 5.10: Transwell invasion and migration assays were analysed after 72 hours' treatment with BaP. Each type of subpopulation is treated with various concentrations of BaP for 72 hours. Cells migrated into the bottom well were quantified with PrestoBlue. Data are expressed as fold changes compared to negative control (DMSO). Statistically significant differences are shown for comparison between BaP treated and negative control samples (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). One-way ANOVA was performed separately to investigate the effect of BaP concentration on the invasiveness and migratory ability of treated sorted and unsorted populations, respectively, at 72 hours.

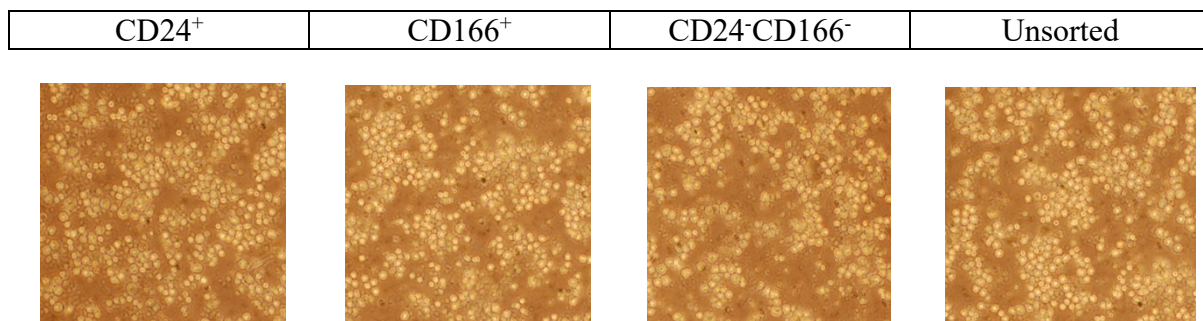


Figure 5.11: Cropped section images of Matrigel that show each type of population treated with the highest BaP concentration (10⁻⁵ M) at 72 hours (images are taken at x10 magnification).

5.3.3.2 *BaP effects on EMT-induce genes– C-MYC, and CTNNB1 in the SNU-1 subpopulations*

As shown in figure 5.12, amongst the sorted population, CD24⁺ is the only population that showed significant induction of *C-MYC* and *CTNNB1* with the treatment of BaP. Elevated level of *C-MYC* mRNA was detected at high BaP concentrations (10^{-8} to 10^{-6} M) at 72 hours. Similarly, *CTNNB1* showed increased expression at 72 hours, and this effect appeared to be dose-dependent with BaP treatment.

On the other hand, there was little change in expression of *C-MYC* with all BaP concentrations at all time-points in CD166⁺ and CD24⁻CD166⁻ sorted populations. Interestingly, decreased expression of *CTNNB1* was observed in both populations treated with BaP at 48 and 72 hours.

The unsorted SNU-1 population, which served as a benchmark for the expression of *C-MYC* and *CTNNB1*, showed increased expression in a dose-dependent manner after 48 hours treatment with BaP. Overall, this finding is a piece of further evidence to support my previous speculation that CD24⁺ is the most plastic population amongst the sorted SNU-1 cells (see page 111).

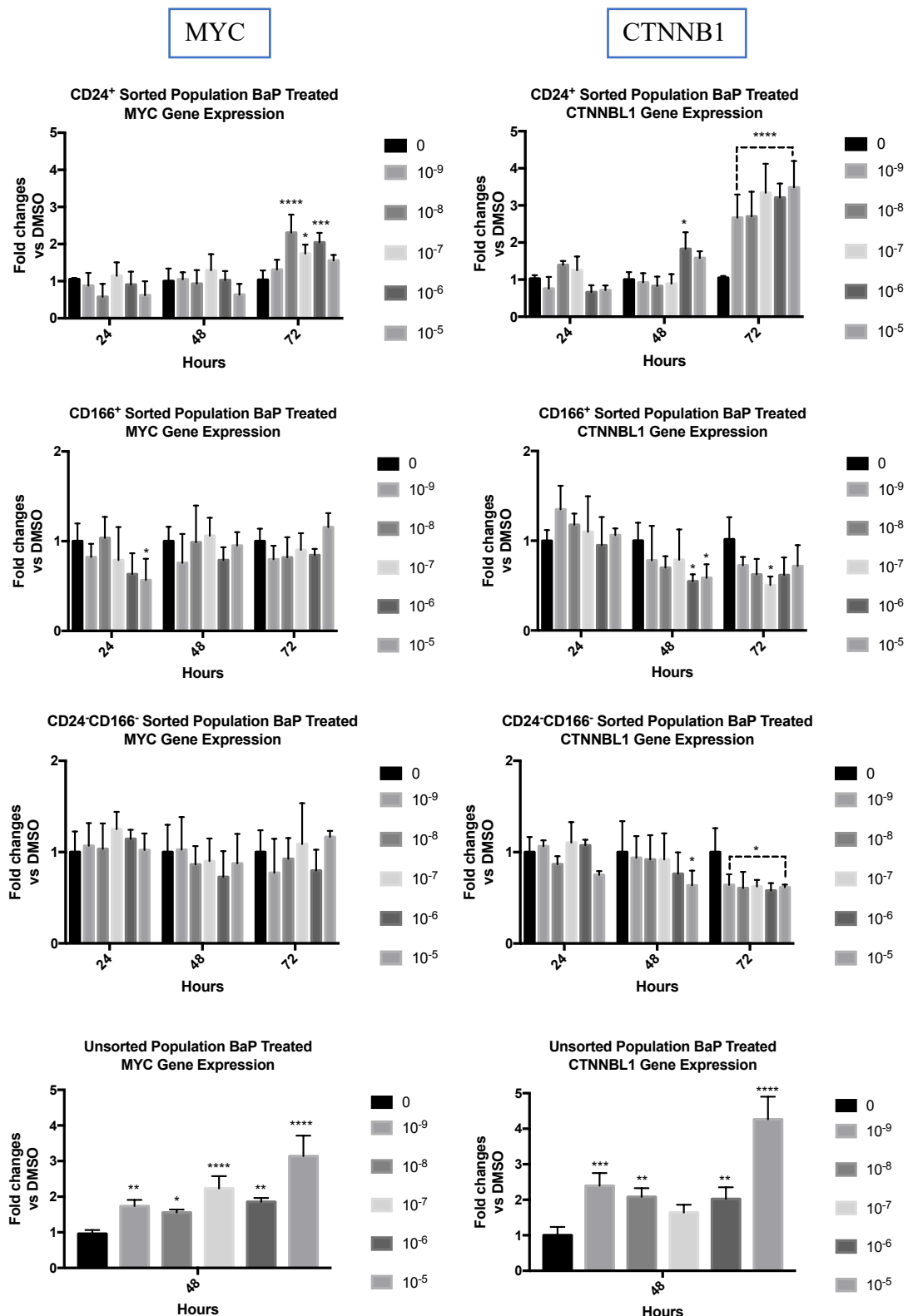


Figure 5.12: Data is shown for sorted, vs. unsorted SNU-1 populations at 48 hours. C-MYC expression is largely associated with cancer formation and progression, whilst CTNNB1 expression is closely associated with B-catenin and WNT-signalling pathways and linked with migratory and invasive ability of cancer cells and carcinogenesis. This data showed different sub-populations' response to BaP treatment, which represents the level of plasticity of each sorted population that leads to phenotypic change. Statistically significant differences are shown for comparison between BaP treated and negative control samples (DMSO). Values are $\bar{x} \pm SD$, $n = 4$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Two-way Repeated Measures ANOVA with Time and BaP concentration as independent variables was performed separately to check whether BaP concentration has an effect MYC and CTNNB1 gene expression in sorted and unsorted populations, respectively.

5.4 Discussion

The AhR plays an important role in the induction of xenobiotic metabolism and is known to be involved in various cellular signal transduction pathways [199], [236], [291] such as epidermal growth factor receptor (EGFR) [292], [293], NF-kB signalling, and protein kinase A (PKA) [294]. On the other hand, BaP is a potent ligand of *AhR*, and its ability to lead the activation of *AhR* gene expression is known. Here, I investigated the response of SNU-1 subpopulations to BaP treatment and the biological consequences of the treatment: (1) BaP induced differential phenotypic response in the subpopulations of SNU-1 gastric cancer cell line, and (2) each subpopulation showed different level of plasticity in response to BaP treatment.

Potent polycyclic aromatic hydrocarbons (PAHs) such as BaP are strong ligands for *AhR* which can activate the ligand-activated transcription factor. Upon activation, the transcription factor regulates target genes like *CYP1A1* and *CYP1B1*, which subsequently participate in the metabolic process of the introduced xenobiotic. However, the role of *AhR* repressor, *AhRR*, is ubiquitous in this process with regulatory roles in inflammatory response and cancer biology [295]. However, there are strong indications supporting the hypothesis that the toxicity and carcinogenic action of dioxins cannot completely be explained by the classical *AhR* signalling pathway (CYP family *1A1* and *1B1*) alone [259] [296] [297]. Highly expressed or overexpressed *AhRR* has a major impact on the regulation of acute toxicity of *AhR* ligands [297] and impacted the responses of skin and intestine, bone-marrow derived macrophages upon reaction to the ligand's toxicity effect [296]. These findings highlight the importance of *AhR* and *AhRR* expression in a cell type-specific manner in response to environmental stimuli.

In this study, *AhR* and/or *AhRR* are highly expressed in CD24⁺ cells in response to BaP treatment, compared to other sorted populations. Although AhR protein has structural similarities to AhRR, the latter lacks the established *AhR* ligand capability [298]. Its regulation is possibly governed by a dioxin-responsive element located in the promoter region of the gene, suggesting that *AhR* is controlling the expression of its repressor [299].

These findings provide a possible explanation for the fact that a high expression of *AhR/AhRR* seems to correlate with a high expression of *NANOG* in CD24⁺ population at 72 hours. Surprisingly, CD166⁺ population showed negative activation of *AhR* in response to BaP treatment at 72 hours, and *SOX2* expression appeared undetected even after 40 cycles of RT-

PCR. It is possible that stemness characteristics appeared to be regulated by *AhR/AhRR* expression in both CSC-like populations in response to BaP. In support of this, the population lacking CD24⁺ CD166⁺ (namely, CD24⁻ CD166⁻) showed a correlation between *AhR*, *ARNT*, and *AhRR* expressions with SOX2 and *NANOG* expressions with BaP treatment at 10⁻⁹M at 48 hours.

Furthermore, I investigated the effect of BaP on influencing the expression of the proto-oncogene, *C-MYC*, in the sorted populations of SNU-1. In the transcription factor network, *C-MYC* is the key molecule that regulates various functions. Its activation achieves broad effects causing different physiological consequences in cell survival, cell adhesion, and energy, nucleotide, and amino acid metabolism [300]. It is also known as the most potent oncogene for cell transformation phenotypes [301], [302]. However, *C-MYC* alone generally cannot induce tumorigenesis, rather it acquires the ability to induce neoplastic “transformation” by recruiting other cooperating transcriptional regulators and cofactors (such as the heme group in the CYP family enzymes) [300]. In gastric cancer, *C-MYC* is overexpressed and was found to regulate cellular proliferation, apoptosis and cell differentiation [177], [303]. In my findings, the high expression of *CTNGB1* CD24⁺ alongside *C-MYC* after BaP treatment is a potential indication that the regulation of *MYC* gene expression may work in association with aberrant Wnt/ β -catenin signalling pathway with the BaP treatments. This activation may produce a highly invasive phenotype in the CD24⁺ as observed in figure 5.10. In line with my finding, Li Ma *et al.* 2010 suggested that *MYC* can activate carcinoma cell motility and invasiveness through β -catenin signalling, which contributes to metastasis formation [304]. A similar association was also observed in colorectal cancer and teratocarcinoma cells [305], [306], where oncogenic Wnt signalling and *C-MYC* induce stemness in cells by restraining cellular senescence and differentiation [306], [307], which could encourage EMT [150], [263], [306]. Overexpression of *MYC* in luminal mammary epithelia encouraged the rise of breast CSC with the metastatic potential. It can also cause the cells to acquire a stem-like state by inducing an activation of enhancers and promoting a stem cell-like transcriptional program [308]. Thus, I found that only CD24⁺ population responded to BaP treatment by inducing *C-MYC* upregulation, which may promote increased motility and stemness characteristics. However, CD166⁺ sorted population exhibited similar invasive and migratory characteristics and increased stemness expression when treated with BaP but without *C-MYC* overexpression. EMT-TF genes were also activated differently: in CD24⁺, *Snail* was upregulated whereas in CD166⁺, *TWIST1* was upregulated. However, both upregulations are likely to be causing the invasiveness of these subpopulations.

5.5 Conclusion

In conclusion, this study demonstrates that BaP can promote phenotypic change in the sorted populations, which results in acquired motility and stemness phenotypes. The upregulation of key genes such as *AhR/AhRR* and *C-MYC* provides interesting insights on the possible downstream regulation of the stemness genes. Moreover, miRNA role in this study is also yet to be investigated. It would be interesting to see if epigenetic modifications play a critical role in the metabolic activation of environmental chemicals.

This study is the first to characterize key phenotypic changes in CSC-like cells isolated from total population of SNU-1. Despite the complexity of the CSC models, I believe that the idea that only small cell sub-populations are capable of expressing stemness characteristics is not entirely in line with this study. My data suggest CD24⁺ subpopulations showed the best plasticity and an increased potential to acquire CSC-like phenotype compared to the other sub-populations. Nevertheless, it is certain that phenotypic change is also inducible in other plastic sub-populations such as CD166⁺.

6 General Discussion of the project

6.1 Hypothesis

Throughout this PhD, I have studied the plasticity of gastric cancer stem cells and developed the following hypotheses:

- Stem cell-like populations can be identified and isolated in gastric cancer (GC) cell lines.
- Environmental chemicals phenotypically alter gastric cancer cell lines to promote a stem-like phenotype.

6.1.1 Aim of the study

The overarching goal of this PhD project was to investigate the plasticity of gastric cancer cell lines, when induced with environmental carcinogen. This was achieved in several steps throughout the thesis. Firstly, in Chapter 2, stem cell-like subpopulations were identified and isolated from gastric cancer cell lines. These subpopulations included CD24, CD44, CD133 and CD166. However, the rest of the project focused on sorted populations of CD24 and CD166 in SNU-1 cell line. The reasons for this choice were explained in detail in Chapter 2, section 2.5. Then in Chapter 3, the sorted SNU-1 population was phenotypically characterized with regards to stemness gene expression, and the expressions of essential genes involved in xenobiotic response and EMT. Chapter 4 investigated whether the carcinogen exposure is related to the changes in stemness markers within gastric cancer cells.

Finally, Chapter 5 brought all the previously acquired information together in order to determine the effect of BaP treatment on gastric cancer cell subpopulations, on the expression of stemness markers and on DNA damage.

Gastric cancer (GC) is the third cancer-leading cause of mortality worldwide, causing almost 723,000 deaths annually [309]. It equally affects males and females from different demographics and races from various parts of the world [5]. While *H. pylori* infection is the main contributor to gastric cancer incidence [6], [12], other factors such as unhealthy lifestyle habits and unhealthy diet are also likely to be contributors to gastric cancer [177], [186], [203], [310].

Typically, Lauren classification is the most commonly adopted and accepted method by pathologists and physicians to histologically classify the tumour [9]. The division into intestinal and diffuse-type GCs is based on the differences of pathology, epidemiology, and aetiology exhibited by GC. While patients with severe gastritis and metaplasia develop the intestinal-type tumours [13], patients with diffuse-type GC have poorly differentiated tumour, cellular decohesion and poorer outcome [14]. The poor outcome has always been associated with tumour metastasis, which greatly affects the patient survival rate [79], [170], and with down-regulation and gene mutation of *E-cadherin*, an important prognostic marker for a poor outcome as it is linked to epithelial-mesenchymal transition (EMT) [253], [304], [311]-[313]. Yet, a major caveat of this classification is that it does not account for the heterogeneous nature of the GC cells [15], [41], [124], [314]. Heterogeneity is a commonly accepted concept that, within a tumour population, cancer cells have phenotypic and functional differences in the cellular properties as a consequence of genetic change, environmental differences, and reversible phenotypic change [124], [219].

The central idea of tumour heterogeneity posits that some cancers are hierarchically organized into various subpopulations of tumorigenic cancer stem cells (CSCs) and non-tumorigenic cancer stem cells progeny [97], [124], [158]. The CSC population is thought to be the main driver of the disease progression, therapy resistance, and perhaps metastasis [106], [114], [143]. However, this may not always be the case – in the various literature cited in this thesis, some cancer cells were found to undergo transitional changes that varied in their competence to contribute to tumour growth [106]. Some markers are reversibly turned on and off within lineages of the cells, and exposure to certain microenvironments may promote their ability to form a tumour [136]. These rare subpopulations in cancer cells exhibit resistance to a variety of therapeutics by reversibly forming sensitive or resistant progeny depending on the therapeutic approach [124]. In addition, these cancer cells can also give rise to phenotypically diverse cells that may or may not contribute to the disease progression [91].

6.2 Chapter 2: CSC-like subpopulations can be identified in the GC cell lines.

Based on the idea that rare subpopulations can promote GC growth, many studies attempted to identify this uncommon subset within GC taken from primary source or metastatic sites. By characterising the CSC-like population based on specific cell surface markers such as CD24, CD44, CD166, CD133, Takaishi *et al.* explored the various CSC potential markers in seven gastric cancer cell lines and found that CD44⁺ subpopulation showed not only greater tumour initiating capacity in mice but also resistance to chemotherapy [44]. The subpopulation characterised by the CD markers was also found in various gastric cell lines, and these subpopulations exhibited significantly higher expression of stemness transcription factor of *SOX2*, *OCT3/4*, and *NANOG* [257]. A similar observation was made about pancreatic adenocarcinoma [123], colon [112], and breast [213]; it was seen that these CSC-surface markers-expressing subpopulations showed characteristics for self-renewability and stemness. Therefore, in Chapter 2, I confirmed the characterisation of these cell lines (SNU-1, KATO-III and NCI-N87) with an additional finding on the stemness factors expression. Passage number can affect cell phenotype [32] and the regulation of the expression of cell surface markers [276]. I found that early passage of these GC cell lines showed greater expression of CSC surface markers. Similarly, in ovarian cancer [219], a tumorigenic population could later produce non-tumorigenic CD133⁻ cells with increasing passage. The importance of this finding that cell phenotype changes with passage numbers may possibly explain the various inconsistencies among studies investigating the CSC subpopulations within cancer cell lines [105], [315]. Interestingly, I found that CD44 expression was high (94%) in KATO-III cells; this is consistent with the report of Takaishi *et al.* [41]. In a breast cancer study, it was reported that 31% of the tumours have CD44⁺/CD24⁻ subtype and the percentage of cells expressing CD44 varied from 0 to almost 100% [229]. Thus, CSCs could also be as abundant as a non-CSC population. Thus due to the nature of CD44 cell surface glycoprotein, which is involved in cell-adhesion [220], CD44 is likely to be highly present in various adherent cell lines and intestinal-type GC cell lines such as KATO-III. Thus, I decided that CD24 and CD166 from SNU-1 were likely to be the more appropriate markers to be used in my investigations are expressed in the cell line. In addition to that, SNU-1 was my choice of cell line as it was sourced from primary gastric carcinoma, poorly differentiated GC [22], [31] and diffuse-type GC [43]. Therefore, it is possible that expression of these CD markers could play an essential role in the subpopulation's migratory and invasion capability – a key hallmark of cancer, as discussed in Chapter 1 (section 1.7). Pluripotent markers' (*SOX2*, *OCT3/4*, and *NANOG*) expressions are

crucial in the maintenance of CSC and tumour cells. I showed that these pluripotent markers are expressed in SNU-1. My data suggests that the activation of these stemness factors is significant in the manifestation of metastasis *via* epithelial-mesenchymal transition (EMT) [150], [253], [313], [162], [316], [317].

6.3 Chapter 3: CSC-like subpopulations can be isolated from the GC cell line, however their stemness expressions vary within each subpopulation.

Under the assumption that most cancer cells have equal potential to proliferate and metastasize, for a successful treatment, all cancer cells must be eliminated. However, this does not solve the problem of possible post-treatment relapse. In GC, a major problem in diffuse-type GC is the lack of chemotherapy responsiveness in patients, with recent study indicating > 50% rate of relapse within 12 months of receipt of the treatment [318]. Thus, a better understanding of the biology of diffuse-type GC is essential to design better treatment regimens [14]. It has been suggested that CSCs within tumours are responsible for driving the growth and progression of many cancers [114], [169]. [114]. Studies have also indicated that CSCs are more resistant to treatment [44] than non-CSCs and can cause relapse [169], [205]. However, a characteristic of cellular plasticity such as increased stemness potential within CSC and non-CSC cells complicates this. Several studies have showed an adaptive mechanism in stem-like cells within different population pools [145]-[147]. One study investigating ablated *Lgr5*-expression cells in mice induced *via* diphtheria toxin receptor gene showed that the loss of *Lgr5*-expressing cells is compensated by *Bmi1*-expressing stem cells [145]. This is a possible indication of potential reprogramming of dynamic stemness within the cells that can affect their phenotype [136]. In another study, combined induction of stemness *POU3F2 (OCT3) + SOX2 + SALL2 + OLIG2* transcription factors in differentiated glioblastoma cells created tumour initiating cells in 100% of animals studied [137]. Similarly, in colorectal cells, with the induction of *OCT3/4*, *SOX2*, and *KLF4* CSC properties can be enhanced [138]. Recognition of molecular heterogeneity in a subpopulation is important to determine the prospective stemness subpopulation that may have the ability to sustain the disease progression.

In Chapter 3, I showed that enriched populations of the CD24⁺, CD166⁺ and population depleted of CD24 and CD166 all express some level of stemness, and with time their phenotypes change. Stemness factors expressed in these populations may yet play an important role in sustaining the self-renewability of SNU-1 and inducing EMT with the activation of

genes critical to this process. It is possible that these two factors (stemness and EMT) are coupled [256]. As shown in CD166⁺ subpopulation, increased stemness expression of *e.g.* *NANOG* may correlate with the increased expression of *CTNNB1* and *MYC*. Key Wnt signalling gene, *CTNNB1* crosstalk with the expression of stemness factors is also possible [280] with aberrant Wnt- β -catenin activation being a hallmark of cancer, especially in promoting metastatic potential [166], [196], [275]. Some of the EMT-associated genes are shown to be directly activated by *NANOG* [248]. Yet in my studies, CD24⁺ subpopulation showed no correlation between the stemness expression with the EMT-associated gene, in contrary to various studies that show this association with EGF/EGFR signalling (one of signalling pathways in EMT) in GC [110] and with metastasis [213]. Nevertheless, *ABCG2* (a member of ATP-binding cassette (ABC) transporter superfamily) expression was only detectable in the CD24⁺ population (Figure 3.7). *ABCG2* contribution to cell resistance to multidrug treatment in chemotherapy [249] and self-renewal regulation is well documented as a recognised stem cell marker [82], [250] indicating that stem-like plasticity could possibly be exclusively possessed by the CD24⁺ population. It is likely that different levels of cellular plasticity can also be observed within the subpopulations. Therefore, I hypothesized that Aryl hydrocarbon receptor (*AhR*), which can control cell growth by manipulating stemness [189], [199], [239], [291], may serve as a plasticity control mechanism in SNU-1. Thus, it is highly probable that upregulation of multiple stem cell associated genes can be made possible by inducing *AhR* activity. The activation of *AhR* by dietary ligands has a direct impact on intestinal stem cells proliferation [188]. *AhR* activation is critical for exacerbating tumour cell proliferation and metastasis [177], [185], wherein the importance of the receptor role in endowing cells with CSC-like quality in breast cancer was suggested [239].

6.4 Chapter 4: BaP induced molecular genotoxicity and plasticity to SNU-1 via *AhR*-mediated response.

As an *AhR* agonist, BaP is known to induce *AhR* activation resulting in the upregulation of gene transcriptional targets such *CYP1A1* and *CYP1B1* [236], [282]. The ultimate metabolic consequence of the BaP was confirmed with the formation of micronuclei in the SNU-1 treated with BaP after 72 hours of treatment (Refer to Chapter 4, Figure 4.4). However, the cascade of events triggered by the BaP exposure appeared to affect various phenotypic responses in the cell line across the 72 hours of culture. The most prominent BaP-mediated responses observed throughout the chapter 4 are:

- 1) *AhR* activation induces CYP1 family regulation at 48 hours;
- 2) The increased expression of surface protein marker of CD24 and CD166 at 48 hours;
- 3) Stemness genes' upregulation begins at 24 hours and changes with time;
- 4) EMT-transcription factor, *Snail*, was upregulated at 72 hours inducing similar upregulation of EMT-inducer genes, *MYC* and *CTNNB1*;
- 5) Induced cell motility resulted in the increased number of cells invading the Matrigel membrane.

AhR transcriptional targets such as *CYP1A1* and *CYP1B1* are shown to be the result of the direct activation by *AhR* [279]. Metidji *et al.* (2018) suggests that *AhR* can also act as a regulator of the Wnt- β -catenin pathway by regulating key genes in the pathway [188]. Similarly, the activation of β -catenin enhanced the transactivation¹⁵ potential of ligand-activated *AhR* at DNA level, where coimmunoprecipitation confirmed a physical interaction between *AhR* and β -catenin [319]. In my experiments, I suspect that the crosstalk between *CTNNB1* (key activator of the Wnt signaling pathway) and *AhR* enhanced the stemness characteristics of SNU-1 and promoted cell invasion of the Matrigel following BaP treatment.

Thus, I propose that following BaP introduction to the culture (refer figure 6.1), *AhR*-Wnt signalling up-regulates the activity of the *Pou5f1* (*OCT3/4*) distal enhancer via *SOX* motif [280]. BaP-mediated response was sufficient to cause perturbation to *CDH1* (the gene encoding E-cadherin protein), which triggered EMT via *Snail* and *CTNNAL1* [320] in the Wnt- β -catenin signalling pathway (by upregulation of *CTNNB1* at 72 hours treatment). Genes that are co-

¹⁵ activation of a gene at one locus by the presence of a particular gene at another locus (Oxford dictionaries).

expressed or interact with CTNNB1 activation such as ALCAM (CD166) and CD24 showed increased both gene and protein expressions, which is another possible outcome of BaP-mediated phenotypic response. In addition to the possible interaction or cross-talk between the stemness genes, *NANOG* activation and binding to the promoter region of EMT-associated genes such as *Snail* [248] was also made possible with BaP induction. Although SNU-1 lacks the protein expression of *E-cadherin* due to methylation at the promoter region, using a demethylating agent such as 5-aza-2'-deoxycytidine restores *E-cadherin* expression in SNU-1 [31]. BaP has been shown to restore methylated promoter regions (in the demethylation process) [281]. Thus, I suspect that the treatment with BaP may have altered the methylation at the DNA promoter of *CDH1* to potentially enhance EMT transformation of SNU-1 cells to acquire a more migratory and invasive phenotype (refer to Figure 6.1).

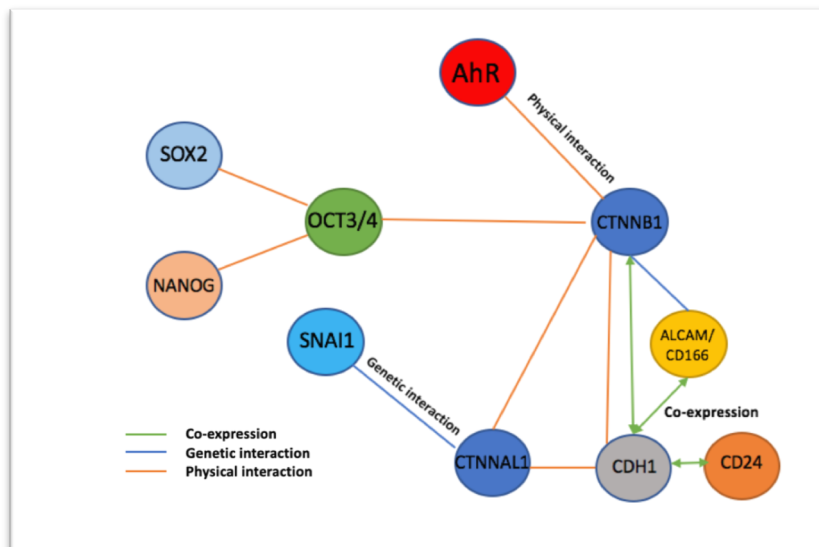


Figure 6.1: Predicted protein interactions. Data generated from GeneMANIA.org [78]. (Given a query of several gene list, GeneMANIA finds functionally related genes using various genomics and proteomics data in its database. It then weights each functional genomic dataset according to its predictive value and generates potential relationships between genes.)

In this thesis, I hypothesized that BaP can induce phenotypic change to SNU-1 populations. Thus, BaP-mediated effects were confirmed with the early genes' expression analysis (with QRT-PCR), and the frequency of micronuclei formation on the treated cells or subpopulations (with micronucleus (MN) assay). In chapter 4, the early genes' expression analysis showed increased expression of *AhR* and *ARNT* genes [179], [180], which signifies potential increase of gene transcription to protein. These findings corroborate with the established studies regarding BaP molecular activation within cells, as the AHR binds to ARNT to form a

heterodimer and translocate into the nucleus, triggering upstream activation of the xenobiotic response elements genes such *CYP1A1* and *CYP1B1*, which I showed to have increased expression following the BaP treatment. The enzymes produced by these CYP1 family genes are responsible for metabolizing BaP to polar metabolites that can be excreted out from the cells. However, this metabolic activation also includes production of the clastogenic mutagens such as BDPE. In chapter 4 (page 107-108), I explained at length the possible impact of the carcinogenic by-product on the cells, which includes micronucleus formation. The MN assays further confirm that the BaP clastogenic endpoint was exerted in the SNU-1 cell line with the formation of micronucleus (refer figure 6.2).

It is important to note that the phenotypic responses caused by BaP treatment, as observed in SNU-1, or SNU-1 subpopulations (investigated further in chapter 5), were possibly enhanced with *AhR*-BaP activation, especially since *AhR* is a direct target of or ligand for BaP [177]. As mentioned in section 1.8.3.1, the *AhR* gene encodes a protein that plays a significant role in many biological functions [175], [188]-[192]. Critically, one of the important findings in this chapter proved that the manipulation of a cell's plasticity (that allow for various phenotypic changes) can be achieved by providing a suitable ligand to this important receptor. However, it is crucial to note that in SNU-1 heterogenous populations, many populations within the cell line do not respond equally to BaP, which I further proved in Chapter 5.

Metabolic activation of BaP that leads to DNA damage

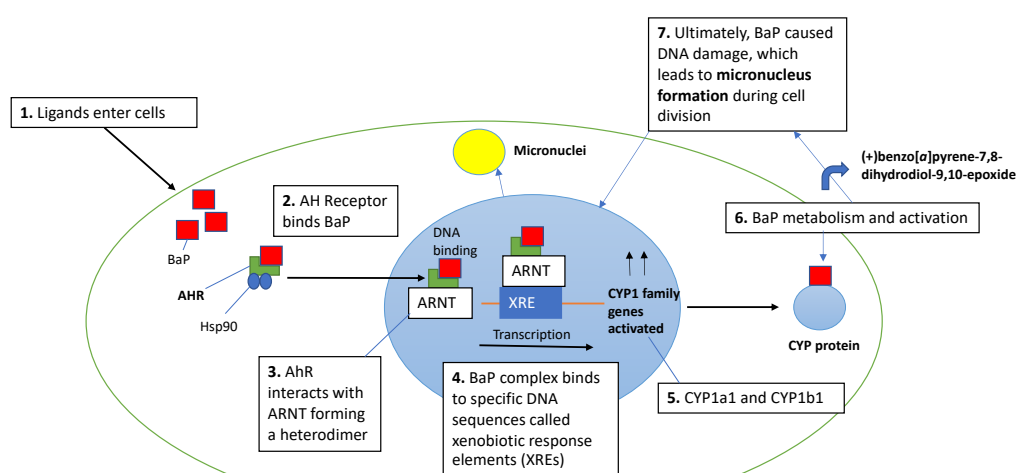


Figure 6.2: With the introduction of Benzo[a]pyrene (BaP) [226] as ligand, it binds to Aryl hydrocarbon Receptor (AhR) and form an AhR receptor complex (AhR + BaP). This complex translocate into nucleus and formed a heterodimer with Aryl hydrocarbon Nuclear Translocator (ARNT) [228], [229] to recognize a XRE binding site (Xenobiotic response element) [230], located at the upstream regulatory region of gene targets, such as cytochrome P450 such as *CYP1A1* and *CYP1B1* [229], [231]. BaP metabolism product by CYPs and epoxide hydrolase resulted in mutagenic and carcinogenic metabolites of B[a]p-7,8-dihydrodiol-9,10-epoxide (BPDE). BPDE can covalently bind DNA guanine residues to form pre-mutagenic adducts caused the formation of micronucleus [232], [233]

6.5 Chapter 5: BaP Induced Plasticity of Sub-populations of SNU-1 Gastric Cancer Cell Line

By using a specific surface marker expression, cancer stem cells *in vivo* and *in vitro* can be sorted into phenotypically distinct subpopulations. From the hypothesis of phenotypic switching, it can be inferred that cancer cells can transform stochastically¹⁶ into cancer stem cells. Gupta *et al.* 2011 showed that sorted cancer cell subpopulations can revert to the original population proportions over time [321]. By using Markov model, they showed that, given certain conditions, breast cancer stem-like cells can switch to non-CSCs, and that there is a probabilistic chance that non-CSC breast cancer cells can switch to CSC-like cells. Other studies do not support this hypothesis. For example, in melanoma, ABCB5⁻ cells do not generate ABCB5⁺ cells [322], and CD34⁺p75⁻ MPCs can produce heterogenous progeny after multiple passages in vitro, whereas CD34⁺p75⁻ MPCs cannot [323]. However, Roesch *et al.* [99] showed that JARID1B⁻ cells can become JARID1B⁺ over time, and Quintana *et al.* showed, using various markers such as CD44, CD166 *etc*, that enriched fractions of cells can revert to the heterogeneity of the tumours from which they were sorted [97]. Thus, in chapter 3, I showed that sorted SNU-1 subpopulations of CD24⁺ and CD166⁺ lost the surface expression in just 72 hours in culture. Moreover, with BaP-mediated plasticity, the surface protein expression was not only maintained but enhanced, and the effect appeared to be BaP dose-dependent. However, this effect was only seen in CD24⁺ cells; strikingly, mRNA expression of CD24 but not CD166 was attenuated in CD24⁻CD166⁻ population, hinting that non-CD24 can give rise to CD24⁺ cells. Thus, the proposed hypothesis mentioned earlier that phenotypes in cancer cell population are not static but reversible in a stochastic manner [97], [142], [321] is supported by my study. I suspect that the alteration in signaling systems in the subpopulations (due to BaP-mediated persistent *AhR* activation and BaP causing genetic lesion) may be perturbed in such a way that cells yield a certain biological response such as increased level of plasticity and induced phenotypic change.

A second conclusion from this chapter is that both CD24 and CD166 subpopulations showed varied response to BaP. I speculated that *AhR* ligand specificity and affinity may vary between

¹⁶ having a random probability distribution or pattern that may be analysed statistically but may not be predicted precisely (Oxford dictionaries).

subpopulations. Interestingly, BaP suppressed stemness expression in CD166 cells, with *SOX2* being completely repressed and rendered undetectable at 72 hours. This supports a report in which BaP treatment of placenta-derived mesenchymal stem cells suppressed the expression level of pluripotency marker, *SOX2*, compared to control, and disrupted differentiation [324]. BaP also induced increased invasiveness of CD166 cells in Matrigel, however it showed no effect in a migration assay. In addition, *TWIST1* was significantly activated with BaP treatment but not *Snail*. In summary, the *AhR*-dependent expression triggers *NANOG* activation and binding to the promoter region of EMT-associated genes such as *Snail* [248]. EMT-associated genes promote stemness phenotype, which renders tumour cells increasingly motile, and also triggers *MYC* and *CTNNB1*, which activate the Wnt- β -signalling pathway, and produce β -Catenin and CD24 expression. In the nucleus, β -Catenin associates with Wnt- β -catenin transcription factors activating target genes such as *AhR* and *Cyp1A1*. Upon activation by a ligand (BaP), the *AhR*-ARNT dimer binds to XREs (xenobiotic response elements) and triggers *CYP1A1* transcription. β -Catenin cooperatively aids *AhR*/ARNT/ XRE-dependent transcription possibly by direct interaction [319]. These interactions are shown in Figure 6.3.

Therefore, in the final results chapter, I demonstrated that BaP can promote phenotypic change in the sorted populations, in particular by inducing plasticity, which causes changes of the phenotypic expression of the surface protein markers and promotes aggressive phenotypes in all the subpopulations, however with the advantage towards CD24⁺ subpopulation (refer to Figure 6.3). Collectively, these observations support the suggestion that environmental chemicals such as BaP can influence the plasticity of cells. My mechanistic studies in these model cell culture systems suggest that plasticity and the potential to acquire CSC-like phenotypes is inducible in the SNU-1 gastric cancer cell line.

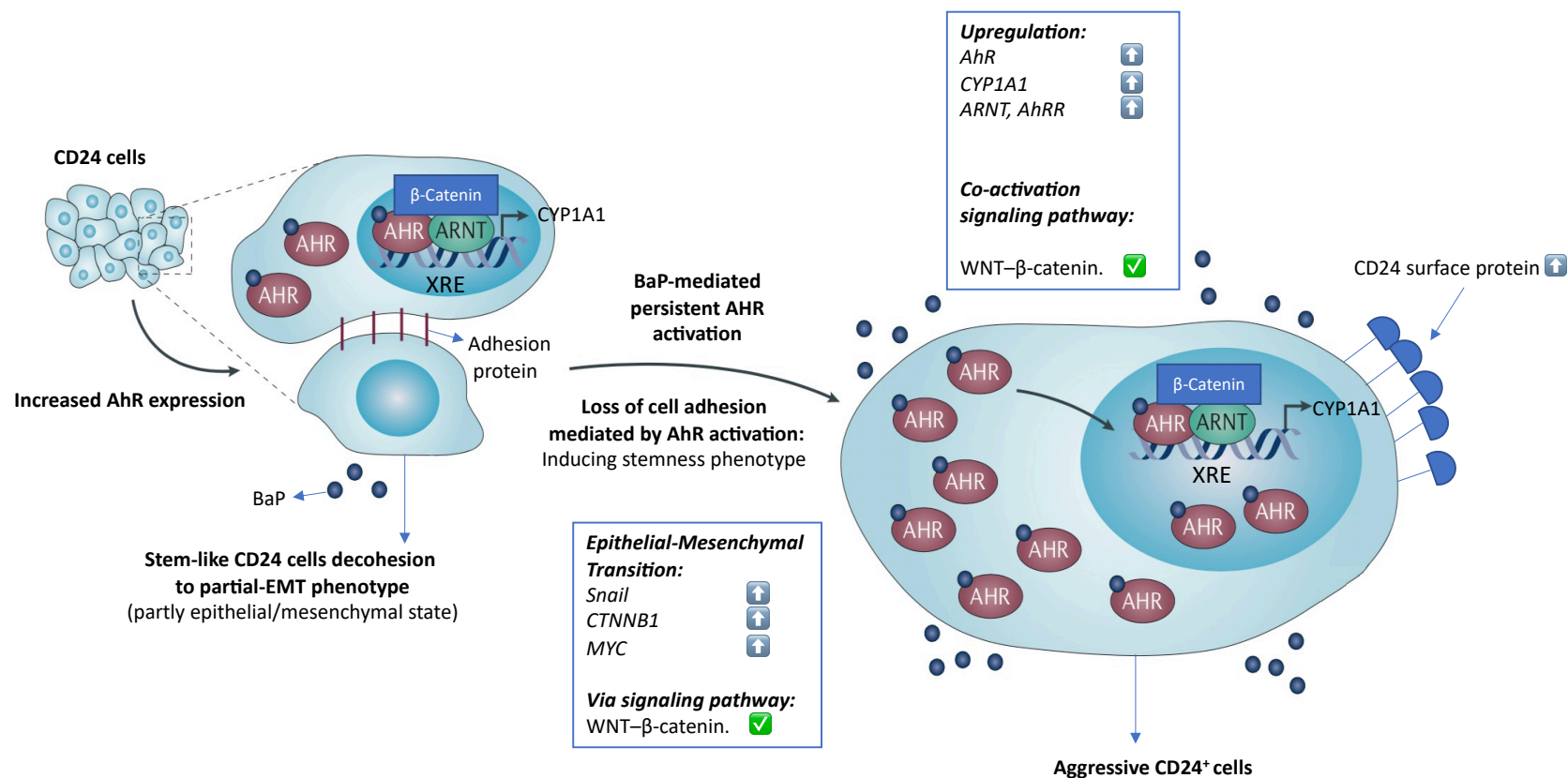


Figure 6.3: Localized AhR agonist, BaP, increases AhR activity, leading to CYP1 activation, the initiation of epithelial–mesenchymal transition and facilitating tumour cell migration and invasiveness. Heightened AhR activity promotes the expression of Snail, which then inhibits E-cadherin expression, thus decreasing cell adhesion. The AhR-dependent expression triggers NANOG activation and binding to the promoter region of EMT-associated genes such as Snail [248], which promotes stemness phenotype that renders tumour cells increasingly motile. BaP-mediated responses later also trigger MYC and CTNNB1 that activate the Wnt-β-signalling pathway and produce β-Catenin and CD24 expression. In the nucleus, β-Catenin associates with Wnt-β-catenin transcription factors activating target genes such as AhR and Cyp1A1. Upon activation by a ligand (BaP), the AhR-ARNT dimer binds to XREs (xenobiotic response elements) and triggers CYP1A1 transcription. β-Catenin cooperatively aids AhR/ARNT/ XRE-dependent transcription possibly by direct interaction [319].

7 Final conclusion

The molecular response elicited by BaP treatment of SNU-1 subpopulations, and the extent of phenotypic change the chemical induces to the plasticity of the gastric cell line have not been studied before. The main hypotheses investigated in this project were:

- i) **A stem cell-like population can be identified and isolated in gastric cancer (GC) cell lines.**
- ii) **Environmental chemicals phenotypically alter gastric cancer cell lines to promote a stem-like phenotype.**

In Chapter 2, subpopulations from SNU-1, KATO-III, and NCI-N87 cell lines have been characterised with CD24, CD166, CD44 and CD133 surface marker expressions. Importantly, early passage cells were found to yield higher expressions of their dominant expressing markers than cells from later passage. Pluripotency markers such as *SOX2*, *OCT3/4* and *NANOG* were found to be expressed in SNU-1 and KATO-III. In Chapter 3, expression of stemness markers, xenobiotic response genes (*AhR*, *AhRR*, *ARNT*, *CYP1A1*, *CYP1B1*), and EMT-inducers genes (*Snail*, *MYC*, *CTNNB1*) were inspected in isolated SNU-1 subpopulations of CD24 and CD166. In unperturbed system, stemness genes were expressed in all subpopulations, including the CD24⁻CD166⁻ population. A notable observation is that non-CD24 and non-CD166 cells (denoted as CD24⁻CD166⁻) are able to produce CD24⁺ and CD166⁺ after some time in culture. They also revert back to their original proportions, as found in the unsorted SNU-1. In contrast, the CD24 and CD166 expressions in enriched CD24⁺ and CD166⁺ respective cells were found to decrease to ~10-15% of initial levels after 72 hours. In Chapter 4, with BaP treatment, the unsorted SNU-1 cells showed increased expression of stemness, and EMT-inducer gene – *Snail*. BaP was also found to activate *CYP1B1* via *AhR*, and the genotoxic response of the chemical was observed by the increased formation of micronuclei in the treated samples compared to the negative controls. BaP mediated response in SNU-1 also included changes to aggressive phenotype, which enhanced the invasiveness and migratory capabilities of the treated SNU-1 cells. In Chapter 5, when BaP treatment was tested on sorted subpopulations, each sorted population of CD24⁺ and CD166⁺ showed different molecular response to the genotoxin. While unsorted SNU-1, and CD24⁺ subpopulation showed a high

frequency of micronuclei formation, CD166⁺ and CD24⁺CD166⁻ populations did not. It was suspected that *AhR* activation played a major role in this observation as the heightened *AhR* activation was found to be parallel to the activation of *CYP1A1* expression in CD24⁺ subpopulation. The sorted populations also responded to BaP treatment by maintaining their surface protein expression of CD24 and CD166 respectively. The induction of enhanced cell motility was also seen in both sorted populations; while CD24 was endowed with increased migratory and invasiveness capabilities, CD166 was found to be only invasive and not migrative. Stemness regulation in both subpopulations was suspected to be causal to these observed effects. Based on the literature research, it was found that NANOG upregulation directly activates *Snail* in EMT induction, whereas SOX2 repression was found to induce MET in cancer cells. Likewise, *Snail* was highly expressed in BaP-treated CD24⁺ subpopulation, whereas TWIST was highly expressed in CD166⁺ subpopulation. Taken altogether, I showed that stem-like population can be isolated from SNU-1 gastric cancer cell line. Secondly, I showed that the potent genotoxins and carcinogens such as BaP are able to enhance the plasticity of sorted subpopulations and induce phenotypic change.

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9 Appendix

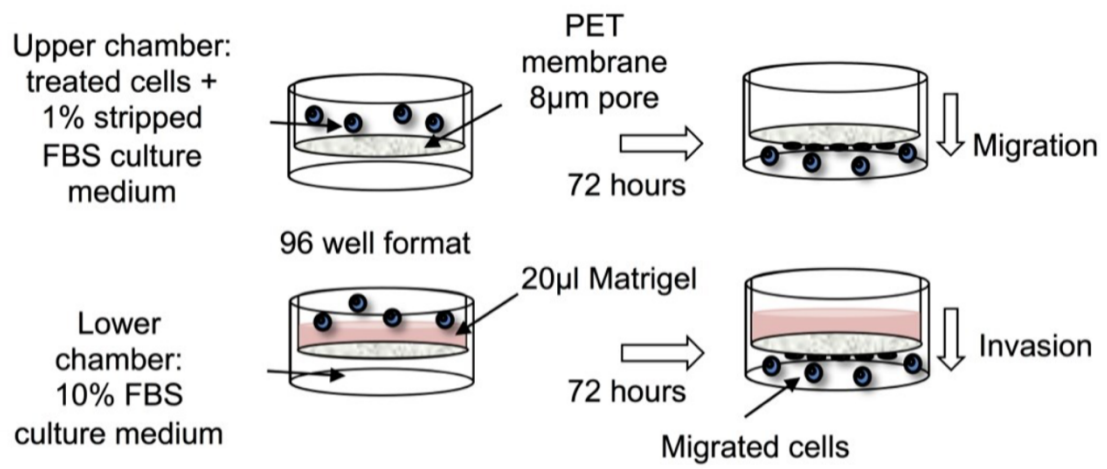
9.1 Primer sequences

Primer sequences used for quantitative RT-PCR assays in this thesis. TaqMan® Gene

Expression Assays (Thermofisher) were used as follows:

Gene type	Gene name	Target species	Gene ID
Normalisation control	GAPDH	human	Hs02758991_g1
Stemness	SOX2	human	Hs01053049_s1
Stemness	POU5F1 (OCT3/4)	human	Hs04260367_gH
Stemness	NANOG	human	Hs04260366_g1
Stemness	ABCG2	human	Hs00184979_m1
Stemness	ALDH1A1	human	Hs00946916_m1
Stemness	LGR5	human	Hs00969422_m1
Xenobiotic response	AHR	human	Hs00169233_m1
Xenobiotic response	AHRR	human	Hs01005075_m1
Xenobiotic response	CYP1A1	human	Hs01054794_m1
Xenobiotic response	Cyp1b1	human	Mm00487229_m1
Xenobiotic response	ARNT	human	Hs01121918_m1
EMT-Inducer genes	SNAI1	human	Hs00195591_m1
EMT-Inducer genes	CTNNB1	human	Hs00355045_m1
EMT-Inducer genes	MYC	human	Hs99999003_m1
EMT-Inducer genes	SHH	human	Hs00179843_m1

9.2 Transwell migration and invasion assay method



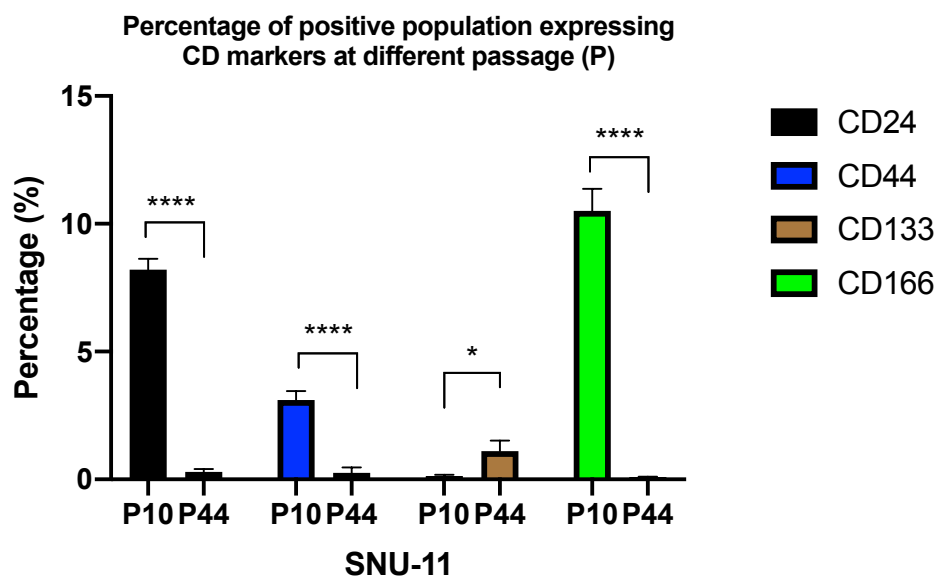
9.3 Surface marker expression of CD24, CD44, CD133, and CD166 on gastric cancer cell lines.

1a: Two-way ANOVA results for SNU-1 cell line:

Table Analyzed	SNU-1				
Two-way RM ANOVA	Matching: Stacked				
Assume sphericity?	Yes				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Passage x CD Markers	32.73	<0.0001	****	Yes	
Passage	42.50	<0.0001	****	Yes	
CD Markers	24.05	<0.0001	****	Yes	
SNU-1	0.4213	0.3215	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Passage x CD Markers	117.3	3	39.09	F (3, 8) = 290.8	P<0.0001
Passage	152.3	1	152.3	F (1, 8) = 1133	P<0.0001
CD Markers	86.16	3	28.72	F (3, 8) = 152.2	P<0.0001
SNU-1	1.510	8	0.1887	F (8, 8) = 1.404	P=0.3215
Residual	1.076	8	0.1344		
Difference between row means					
Mean of P10	5.475				
Mean of P44	0.4375				
Difference between means	5.038				
SE of difference	0.1497				
95% CI of difference	4.692 to 5.383				
Data summary					
Number of columns (CD Markers)	4				
Number of rows (Passage)	2				
Number of subjects (SNU-1)	12				
Number of missing values	0				

1b: Multiple comparison of SNU-1 CD markers:

Compare each cell mean with the other cell mean in that column								
Number of families	1							
Number of comparisons per family	4							
Alpha	0.05							
Bonferroni's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
P10 - P44								
CD24	7.900	6.940 to 8.860	Yes	****	<0.0001			
CD44	2.850	1.890 to 3.810	Yes	****	<0.0001			
CD133	-1.000	-1.960 to -0.04022	Yes	*	0.0409			
CD166	10.40	9.440 to 11.36	Yes	****	<0.0001			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	t	DF
P10 - P44								
CD24	8.200	0.3000	7.900	0.2994	3	3	26.39	8.000
CD44	3.100	0.2500	2.850	0.2994	3	3	9.520	8.000
CD133	0.1000	1.100	-1.000	0.2994	3	3	3.340	8.000
CD166	10.50	0.1000	10.40	0.2994	3	3	34.74	8.000

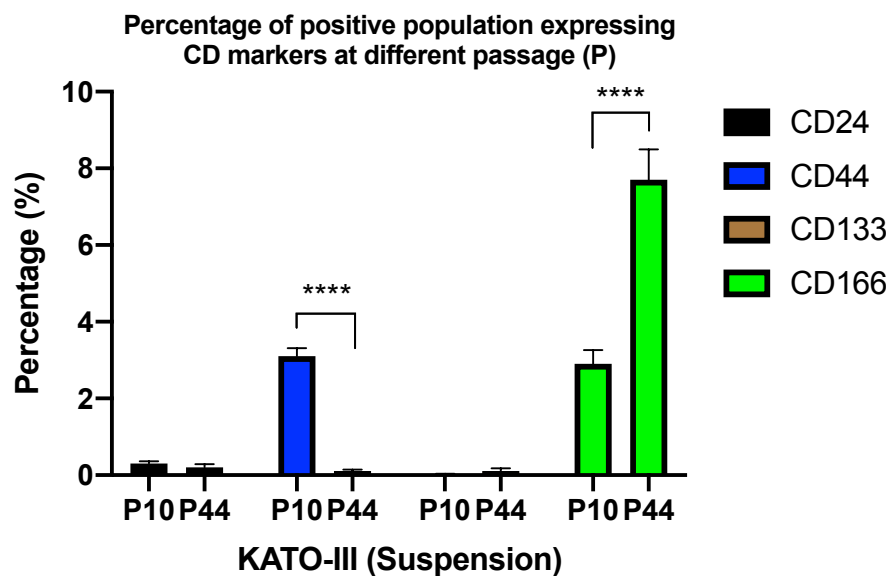


2a: Two-way ANOVA results for KATO-III (Suspension) cell line:

Table Analyzed	KATO-III (Suspension)				
Two-way RM ANOVA	Matching: Stacked				
Assume sphericity?	Yes				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Passage x CD Markers	30.08	<0.0001	****	Yes	
Passage	0.7534	0.0107	*	Yes	
CD Markers	68.11	<0.0001	****	Yes	
SNU-1	0.5071	0.5436	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Passage x CD Markers	46.91	3	15.64	F (3, 8) = 146.1	P<0.0001
Passage	1.175	1	1.175	F (1, 8) = 10.97	P=0.0107
CD Markers	106.2	3	35.41	F (3, 8) = 358.2	P<0.0001
SNU-1	0.7907	8	0.09884	F (8, 8) = 0.9233	P=0.5436
Residual	0.8564	8	0.1071		
Difference between row means					
Mean of P10	1.583				
Mean of P44	2.025				
Difference between means	-0.4425				
SE of difference	0.1336				
95% CI of difference	-0.7505 to -0.1345				
Data summary					
Number of columns (CD Markers)	4				
Number of rows (Passage)	2				
Number of subjects (SNU-1)	12				
Number of missing values	0				

2b: Multiple comparison of KATO-III (Suspension) CD markers:

Compare each cell mean with the other cell mean in that column								
Number of families	1							
Number of comparisons per family	4							
Alpha	0.05							
Bonferroni's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
P10 - P44								
CD24	0.1000	-0.7565 to 0.9565	No	ns	>0.9999			
CD44	3.000	2.144 to 3.856	Yes	****	<0.0001			
CD133	-0.07000	-0.9265 to 0.7865	No	ns	>0.9999			
CD166	-4.800	-5.656 to -3.944	Yes	****	<0.0001			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	t	DF
P10 - P44								
CD24	0.3000	0.2000	0.1000	0.2671	3	3	0.3743	8.000
CD44	3.100	0.1000	3.000	0.2671	3	3	11.23	8.000
CD133	0.03000	0.1000	-0.07000	0.2671	3	3	0.2620	8.000
CD166	2.900	7.700	-4.800	0.2671	3	3	17.97	8.000

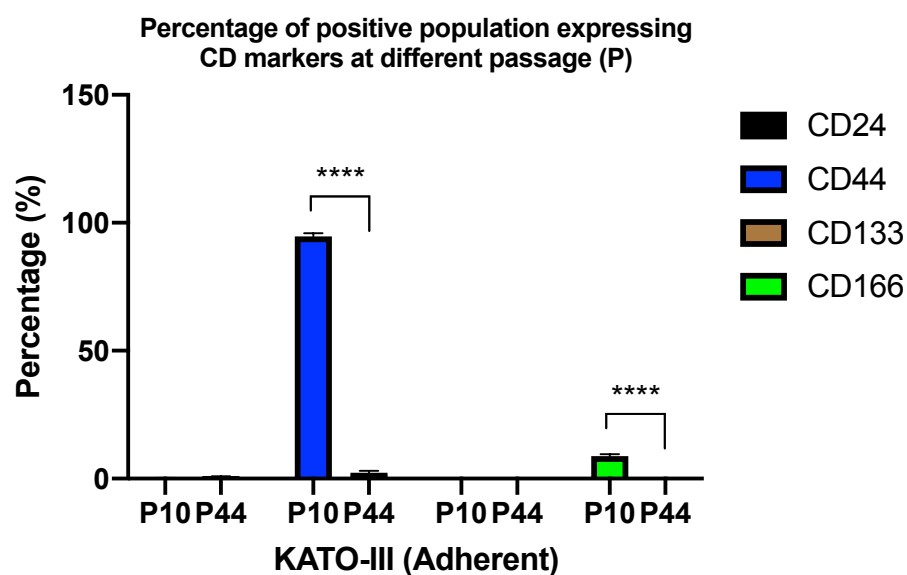


3a: Two-way ANOVA results for KATO-III (Adherent) cell line:

Table Analyzed	KATO-III (Adherent)				
Two-way RM ANOVA	Matching: Stacked				
Assume sphericity?	Yes				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Passage x CD Markers	39.99	<0.0001	****	Yes	
Passage	16.52	<0.0001	****	Yes	
CD Markers	43.46	<0.0001	****	Yes	
SNU-1	0.01986	0.0194	*	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Passage x CD Markers	9140	3	3047	F (3, 8) = 25972	P<0.0001
Passage	3776	1	3776	F (1, 8) = 32191	P<0.0001
CD Markers	9933	3	3311	F (3, 8) = 5835	P<0.0001
SNU-1	4.539	8	0.5674	F (8, 8) = 4.836	P=0.0194
Residual	0.9385	8	0.1173		
Difference between row means					
Mean of P10	25.94				
Mean of P44	0.8533				
Difference between means	25.09				
SE of difference	0.1398				
95% CI of difference	24.77 to 25.41				
Data summary					
Number of columns (CD Markers)	4				
Number of rows (Passage)	2				
Number of subjects (SNU-1)	12				
Number of missing values	0				

3b: Multiple comparison of KATO-III (Adherent) CD markers:

Compare each cell mean with the other cell mean in that column								
Number of families	1							
Number of comparisons per family	4							
Alpha	0.05							
Bonferroni's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
P10 - P44								
CD24	-0.6000	-1.497 to 0.2966	No	ns	0.2569			
CD44	92.40	91.50 to 93.30	Yes	****	<0.0001			
CD133	0.01667	-0.8799 to 0.9132	No	ns	>0.9999			
CD166	8.533	7.637 to 9.430	Yes	****	<0.0001			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	t	DF
P10 - P44								
CD24	0.2000	0.8000	-0.6000	0.2797	3	3	2.146	8.000
CD44	94.70	2.300	92.40	0.2797	3	3	330.4	8.000
CD133	0.03000	0.01333	0.01667	0.2797	3	3	0.05960	8.000
CD166	8.833	0.3000	8.533	0.2797	3	3	30.51	8.000



4a: Two-way ANOVA results for NCI-N87 cell line:

Table Analyzed	NCI-N87				
Two-way RM ANOVA	Matching: Stacked				
Assume sphericity?	Yes				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Passage x CD Markers	1.089	0.1016	ns	No	
Passage	3.317	0.0009	***	Yes	
CD Markers	93.77	<0.0001	****	Yes	
SNU-1	0.8170	0.6101	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Passage x CD Markers	3.330	3	1.110	F (3, 8) = 2.899	P=0.1016
Passage	10.14	1	10.14	F (1, 8) = 26.49	P=0.0009
CD Markers	286.6	3	95.55	F (3, 8) = 306.1	P<0.0001
SNU-1	2.497	8	0.3122	F (8, 8) = 0.8154	P=0.6101
Residual	3.063	8	0.3828		
Difference between row means					
Mean of P10	3.725				
Mean of P44	2.425				
Difference between means	1.300				
SE of difference	0.2526				
95% CI of difference	0.7175 to 1.882				
Data summary					
Number of columns (CD Markers)	4				
Number of rows (Passage)	2				
Number of subjects (SNU-1)	12				
Number of missing values	0				

4b: Multiple comparison of NCI-N87 CD markers:

Compare each cell mean with the other cell mean in that column								
Number of families	1							
Number of comparisons per family	4							
Alpha	0.05							
Bonferroni's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
P10 - P44								
CD24	1.200	-0.4196 to 2.820	No	ns	0.1795			
CD44	0.3000	-1.320 to 1.920	No	ns	>0.9999			
CD133	1.300	-0.3196 to 2.920	No	ns	0.1318			
CD166	2.400	0.7804 to 4.020	Yes	**	0.0058			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	t	DF
P10 - P44								
CD24	3.000	1.800	1.200	0.5052	3	3	2.375	8.000
CD44	0.4000	0.1000	0.3000	0.5052	3	3	0.5938	8.000
CD133	1.400	0.1000	1.300	0.5052	3	3	2.573	8.000
CD166	10.10	7.700	2.400	0.5052	3	3	4.751	8.000

