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RESEARCH ARTICLE

Epithelial-mesenchymal transition and stemness of breast cancer cells: Effect of viscoelasticity of the substrate to mimics microenvironment

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Abstract

Metastasis is one of the greatest challenges in cancer treatment today. Normal mammary epithelial cells are optimally supported by interaction with a soft matrix (microenvironment) with elastic modulus of about 800 Pa. However, after transformation, breast tissue becomes progressively stiffer and tumour cells become significantly more contractile and hyper-responsive to matrix elasticity. In addition, importantly, the cancer cells penetrate into blood vessel and enter the circulation during metastasis. The modulus of fluid such as blood or mucus has very low stiffness of around 50 Pa. For this reason, the critical association between cancer cell phenotype and the change of matrix rigidity with an order of magnitude smaller should be emphasizing. This review highlights the current understanding of epithelial-mesenchymal transition and cancer stem cells in metastasis, and identified importance for investigation on artificial extracellular matrix with different viscoelastic properties, which is required to mimics *in vivo* microenvironment. The substrate damping coefficient ($\tan\delta$) as potential physical parameter emerged the important linkage to cellular motility, cancer stemness, and epithelial-mesenchymal transition induction. Although further investigation is required to clarify the efficacy of environmental stimuli ($\tan\delta$) for tumors exhibiting stem cell-like properties, this review indicates that the cancer cells incubated on softer substrate might lead to express cancer stem cell biomarkers exhibiting high expression.

Keywords: breast cancer, metastasis, epithelial-mesenchymal transition, cancer stem cells, viscoelastic properties, microenvironment

1. Introduction

Breast cancer is one of the most common malignancies in women and is the higher incidence and mortality rates among all female malignant tumors in Japan[1,2]. Over 90% of all breast cancer deaths are the result of metastasis, primarily to the bone, lung, liver and lymph nodes (Figure 1)[3]. Figure 2 shows a complex process containing a series of discrete steps of metastasis: (a) epithelial-mesenchymal transition (EMT), during which cancer cells lose cell-to-cell contact and gain motility; (b) local tissue invasion, which is facilitated by the degradation of extracellular matrix (ECM); (c) intravasation, during which cancer cells penetrate the wall of a blood vessel and enter the circulation; (d) homing that bind to platelets, during which cancer cells must survive within the circulation; (e) extravasation, during which cancer cells pass through the vascular wall and exit the blood stream at distant organs; (f) metastatic niche formation at the metastatic site to create a milieu that is favorable for cancer cell growth[3].

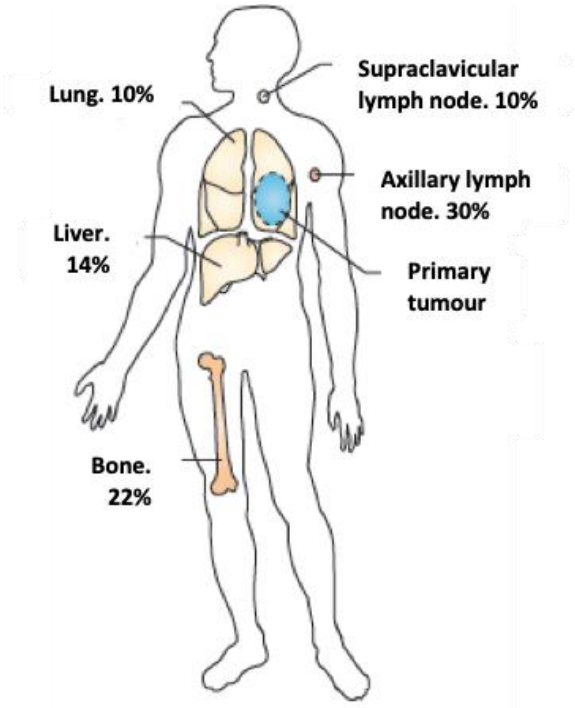

Fig 1: Metastatic spread to different organs of breast cancer. Life-threatening metastases occur most often in the lungs, liver, lymph and bone. (Copyright 2012. Reproduced from ref. 3).

The average 5-year overall survival rate of breast cancer is reported to be around 55% due to the poor outcome of the therapy for metastatic disease in the resistance to radiation and chemotherapy[4]. The breast cancer therapies having poor prognosis prompt us to study an effective cancer therapy. That is, breakthroughs in cancer treatment are essential.

In this review we summarize the current understanding of EMT and cancer stem cells in metastasis, and identify importance for investigation on artificial extracellular matrix (ECM) with different viscoelastic properties, which is required to mimics *in vivo* microenvironment.

2. EMT and cancer stem cell

Normal mammary epithelial cells are optimally supported by interaction with a soft matrix with elastic modulus of about 800 Pa[5]. EMT causes morphological and functional changes in epithelial cells, resulting in the acquisition of mesenchymal cell-like features by cancer cells, where an epigenic program leads epithelial cells to lose their cell-cell and cell-ECM interactions to undergo cytoskeleton reorganization and to gain morphological and functional characteristics of mesenchymal cells (Figure 2a,b). The capability of metastatic cancers to shift motility modes by EMT is one of the main features of invasion. In addition, importantly, the cancer cells penetrate into blood vessel and enter the circulation. The modulus of fluid including blood or mucus have very low stiffness of around 50 Pa (Figure 2). The understanding the interaction between microenvironment and cancer cells is also critical subject to progress in the cancer treatment[6, 7].

A recently proposed hypothesis suggests that cancer stem cells (CSCs) and aforementioned EMT play a pivotal role in cancer metastasis, recurrence and drug resistance[5]. CSCs were first proposed by Virchow and Conheim; a subpopulation of cancer cells resembles the same traits as embryonic cells such as the ability to proliferate, and cancer is derived from the activation of dormant cells of the same tissue. A few CSCs do self-renew and produce a large number of heterogeneous and highly proliferative cancer cells to form primary tumor[8].

Most proliferative cancer cells are killed by chemoradiotherapy. However, CSCs undergoing EMT, which present mesenchymal features, are resistant to anti-cancer therapies. Thus, CSCs survive and cause cancer recurrence. Recent studies showed that inducible factors of EMT not only induce EMT, but also enhance CSC features of breast cancer cells[9]. These findings suggest that EMT is closely related to cancer progression. Therefore, targeting therapy of breast cancer stem cells, it is necessary to investigate the mechanism of EMT.

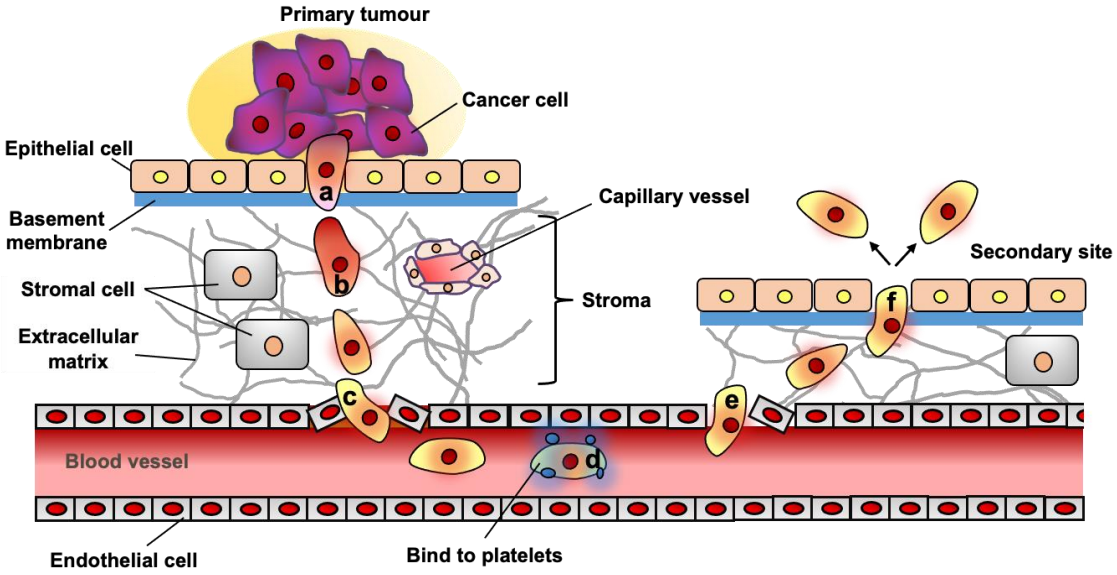

Fig. 2: The several steps of metastasis. (a) epithelial-mesenchymal transition (EMT), during which cancer cells lose cell-to-cell contact and gain motility; (b) local tissue invasion, which is facilitated by the degradation of extracellular matrix (ECM); (c) intravasation, during which cancer cells penetrate the wall of a blood vessel and enter the circulation;(d) homing that bind to metastasis-supporting sites or to platelets, during which cancer cells must survive within the circulation; (e) extravasation, during which cancer cells pass through the vascular wall and exit the blood stream at distant organs; (f) metastatic niche formation at the metastatic site to create a milieu that is favorable for cancer cell growth.
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3. Viscoelasticity and cellular migration

Using polymeric (acrylamide-based copolymer modified with type-I collagen: AC) hydrogel substrates with different viscoelasticity ranged from 30 to 2 GPa as microenvironment of cell culture substrates that influences cancer progression and metastatic potential, Okamoto group have examined the effect of the viscoelasticity on the direct relation between cellular motility and mesenchymal properties with induction of EMT in human breast adenocarcinoma cell line, MCF-7[10].

The viscoelastic features of the substrates (polymeric gel and tissue culture plates (TCP)) are characterized by damping coefficient ($\tan\delta$) and storage modulus (G') (Figure 3). Generally, in the viscoelastic materials, hard and stiff materials such as metals and ceramics show high stiffness and low loss. On the other hand, soft materials have high damping and low stiffness. The solid line indicates a viscoelastic figure of merit (VFOM) $(G'/\rho)\times(\tan\delta)^{0.5}=10^5$ m^2s^{-2}, with ρ as density, for steel, aluminium, poly(methyl methacrylate) (PMMA), TCP (polystyrene, PS), and natural rubber. This was found in traditional solid materials[11,12]. With $\tan\delta$ values of 0.04–0.26, VFOM values of AC gels (~10^{-2} m^2s^{-2}) are seven orders of magnitude lower than that of solid materials. This is achieved by soft matrix with cross-linker molecule. The biomedical properties of a tissue in terms of stiffness (elastic modulus) are shown in Figure 3. Compliant tissue such as lung exhibit low stiffness (300 Pa), whereas tissues exposed to high mechanical loading, such as bone or skeletal muscle exhibit high stiffness with four orders of magnitude greater (10^4–10^6 Pa). By contrast, the blood and mucus exhibit very low modulus of 50 Pa[11,13].

For the diffusion of the seeded cells on the substrates[11], the calculated mean squared displacement (MSD) values and characteristic parameters, cellular migration speed (S), persistent time (P), and cellular diffusivity (D) are presented in Figure 4.

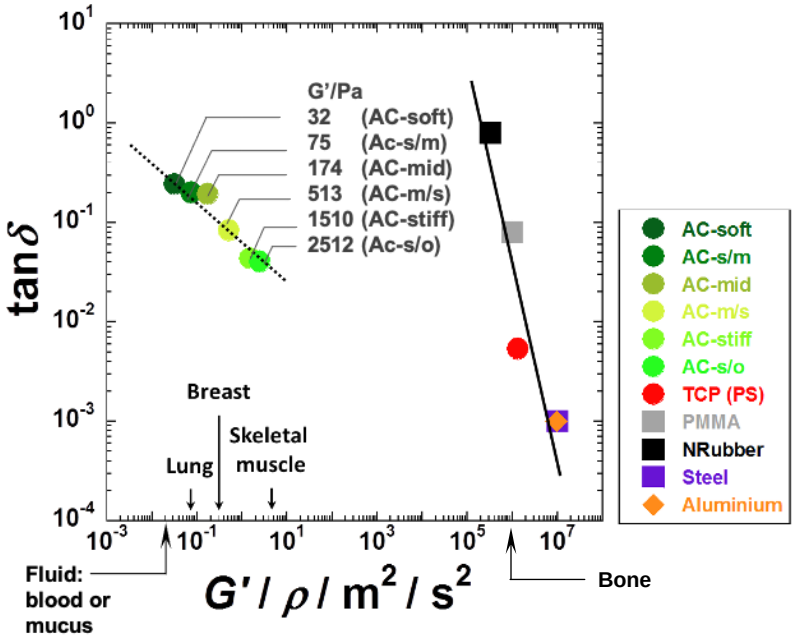

Fig. 3: Damping coefficient ($\tan\delta$) and relative storage modulus (G'/ρ) map for AC gels and various conventional materials at 25°C. The solid line represents a viscoelastic figure of merit (VFOM) $(G'/\rho)\times(\tan\delta)^{0.5}=10^5$ m^2s^{-2}, for steel, aluminium, poly(methyl methacrylate) (PMMA), TCP (PS), and natural rubber[12]. The broken line represents VFOM for AC gels; $(G'/\rho)\times(\tan\delta)^{2.5}=10^{-2}$ m^2s^{-2}. The biomedical properties of a tissue in terms of stiffness (elastic modulus) are shown on the x-axis [13].
(Copyright 2019. Reproduced from ref. 11).

MCF-7 cells cultured on AC-stiff substrate under hypoxia exhibit the highest value of *S* (0.60 μm/min) at day 3, while slight increasing in *S* under normoxic condition (0.67 μm/min) is observed on the same gel substrate. The *S* value of the cells on AC gel substrates under hypoxia changes obviously higher with increasing in gel stiffness (2.4-fold change between AC-stiff and AC-soft) accompanied by an enhancement in the vitality (*P*). The *D* value is the balance between *S* and *P*. AC-stiff substrate exhibits a one order of magnitude larger *D* value in comparison to AC-soft or AC-mid. Overall, *D* is significantly upregulated under both oxygen concentration conditions and the cellular motility on AC-stiff substrate is enhanced. For MCF-7 cells in both normoxia and hypoxia, a slight motility reduction on type-I collagen coated TCP (TCP-coat) of the cells reflects the suppression of *S* and *P*, following the decrease in *D*. In this regard, MCF-7 cells are rather less-invasive cancer cells presumably due to the multicellular aggregation.

Cellular migration speed (*S*) and diffusivity (*D*) in MCF-7 cells under hypoxia is upregulated with decreasing in damping coefficient ($\tan\delta$) (Figure 4b). The stiff substrate increased cellular motility[11]. This result suggests that the decreasing in damping is the driving force of the cellular migration in the cells. In addition, the decreasing in $\tan\delta$ promotes the persistent time (*P*) (Figure 4c). The *D* value (Figure 4d) is important to understand how quick the cancer cells can invade to vasculature or lymph node.

On the other hand, cancer cells destroy the normal balanced in the microenvironment. In addition, during cancer progression cancer cells mainly reconstruct the aberrant extracellular matrix (ECM). The cells do not simply recreate the ECM and the remodeled ECM provides biochemical and biophysical cues to the neighboring cells (cancer cells and stromal cells) to promote cancer progression[14-17].

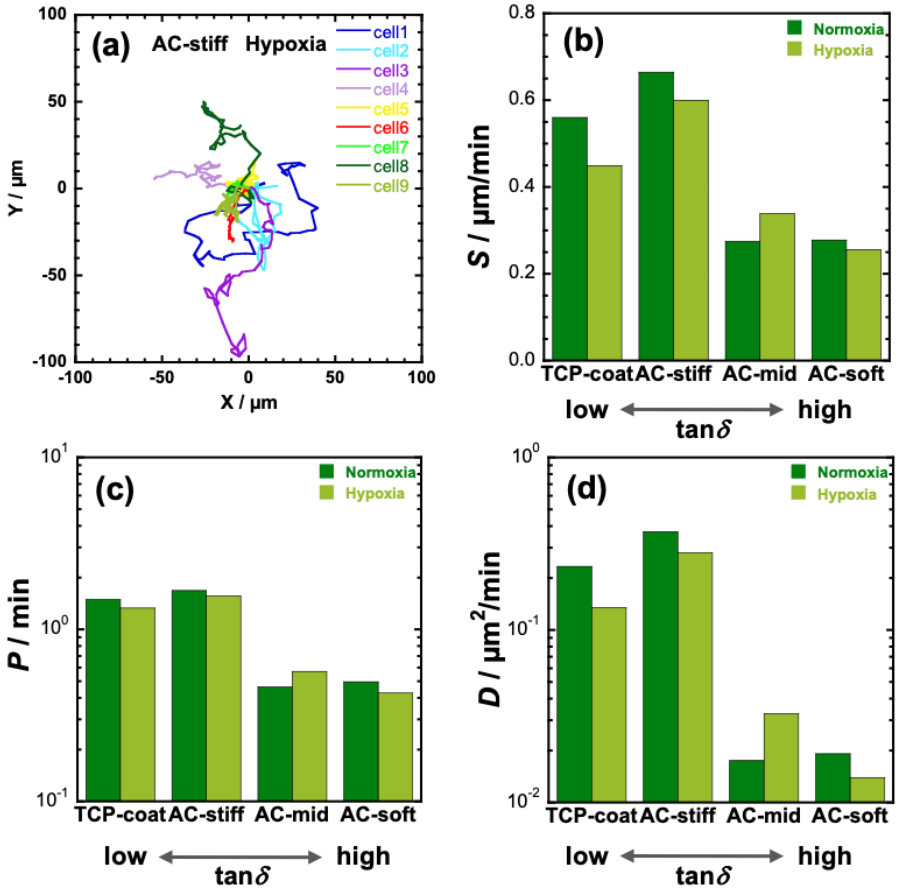

Fig. 4: (a) Trajectory of MCF-7 cells cultured on AC-stiff substrates under hypoxic condition over 16 h at day 3. The data were obtained in 200 x 200 μm^2. Summary of the cellular migration parameters calculated from mean squared displacement (MSD) of MCF-7 cells in both normoxia and hypoxia[11]. (b) cellular migration speed (*S*), (c) persistent time (*P*), and (d) cellular diffusivity (*D*) at day 3 cultured on four different substrates. (Copyright 2019. Reproduced from ref. 11).

Stiffening and aligned extracellular matrices are observed in the vicinity of tumors. Collagen fibers nearby tumor tend to be aligned and cancer cells reorganize collagen fibers to be aligned. This collagen alignment contributes the tumor progression[18,19]. It has been reported that the cancer cells prefer stiffer substrates and the proliferation and motility were enhanced when they were cultured on stiffer substrate[20-24].

4. AC gel substrate contribution to CD133 and CD44 expression

A subpopulation of cancer cells, the CSCs, was found to display stem cell characteristics that influence tumorigenesis[25]. These CSCs have various cancer-promoting characteristics such as self-renewal differentiation, chemoresistance, and metastatic potential[25,26]. CSCs express various CSC markers such as CD133, CD44, and CXCR4 or cells with high aldehyde dehydrogenase (ALDH) activity have been shown to be enriched in CSCs[27]. The expression of CD133 is a strong predictor of declining prognosis, as high CD133 levels conversely relate to low 5-year overall survival and disease free survival rate in cancer patients[28]. Another putative CSC marker is the cell-surface glycoprotein CD44, which was reported to be an adhesion molecule expressed in CSCs[29]. When upregulated, CD44 increases tumor growth and anti-apoptotic property[30]. Both CD133 and CD44 are well-recognized stem cell biomarkers express in breast cancer[31].

For comparison, the expression levels at each substrate tested in this study are plotted as a function of tanδ (Figure 5)[32]. The CD133$^+$CD44$^-$ levels are markedly elevated with increasing in tanδ up to around 0.09 and have a decreasing trend for the cells cultured on AC-soft substrate under both oxygen concentration conditions (Figure 5a). Under normoxic and hypoxic conditions, the CD133$^-$CD44$^+$ levels are promoted by damping coefficient. The level of CD133$^-$CD44$^+$ is over 250-fold for the cells cultured on AC-soft substrate in comparison with that on AC-s/o in normoxia, suggesting that the softer gel substrate produce a

large amount of surface molecule of CD44 (Figure 5b). From these findings, the expression of $CD133^{-}CD44^{+}$ is uncoupled from that of $CD133^{+}CD44^{-}$ in MCF-7 cells under different oxygen concentration levels.

For the co-expression of $CD133^{+}CD44^{+}$, more stem-like properties as compared with other population, seems to be affected by the combination of both $CD133^{+}CD44^{-}$ and $CD133^{-}CD44^{+}$, i.e., a further increase of the levels in the cells incubated on AC-soft ($\tan\delta$=0.244) *via* AC-mid ($\tan\delta$=0.193) substrate is observed (Figure 5c).

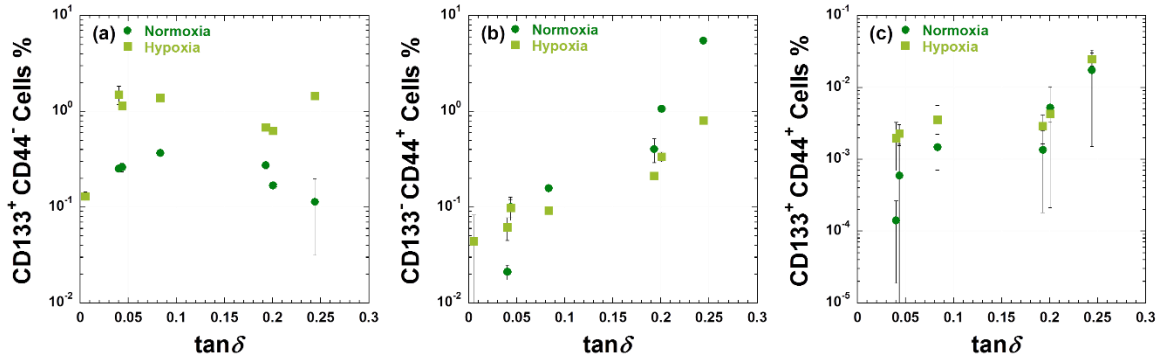

Fig. 5: Relationships between $CD133^{+}CD44^{-}$(a), $CD133^{-}CD44^{+}$ (b), and $CD133^{+}CD44^{+}$ (c) expression and damping coefficient ($\tan\delta$) for MCF-7 cells at day 7 cultured on six different AC gel substrates and TCP-coat under both normoxic and hypoxic conditions. (Copyright 2021. Reproduced from ref. 32).

5. AC gel substrate induced EMT

The gene expression changes have been investigated to understand the role of ECM for malignant phenotype. Typical epithelial cell marker of E-cadherin (CDH1) and mesenchymal marker of vimentin and N-cadherin (CDH2)[33-37] were analyzed because EMT is a critical phenomenon induces cancer metastasis[38-44].

To know detail of EMT and metastasis, transforming growth factor β (TGF-β)[33,44,45] and snail family zinc finger 2 (SNAI2)[33,46,47] and zinc finger E-box binding homeobox 1 (ZEB1)[44,46-49] were added to analysis. TGF-β is known to induce EMT. SNAI2 and ZEB1 are potent repressor of epithelial cell marker CHD1 gene expression. Cancer cells respond to the hypoxic microenvironment through the activity of hypoxia inducible factor 1α (HIF-1α)[50-52] that behaves as a promotor of EMT (Figure 6).

Figure 7 shows the gene expression (HIF-1α, TGF-β, vimentin, CDH2, ZEB1, SNAI2, and CDH1) for MCF-7 cells cultured on AC gel substrates and TCP-coat at day 7 as a function of $\tan\delta$ under both normaxic and hypoxic environment. In both normoxia and hypoxia, MCF-7 cells incubated on AC-soft ($\tan\delta$=0.244) and AC-mid ($\tan\delta$=0.195) substrates show a significant change in HIF-1α expression (Figure 6a). While significant repression is observed for the cells culture on AC-s/m ($\tan\delta$=0.201). For TGF-β level under hypoxic condition, similar change is obtained in MCF-7 cells on all AC gel substrates and TCP-coat (Figure 7b). The cells incubated in the normoxic group do not show a significant increase in TGF-β expression.

For vimentin expression, this surface protein is expressed with similar manner on AC gel substrates and TCP-coat (Figure 7c). The vimentin level under hypoxic condition is markedly increased in the cells incubated on AC-soft (720-fold) and AC-mid (360-fold) in comparison with that of incubation from AC-stiff substrate up to TCP-coat. Similar result is obtained when the cells are incubated in normoxia, while the cells express less vimentin (30-fold) in comparison with AC-soft and AC-stiff substrates.

The expression of CDH2 shows in Figure 7(d). For CDH2 expression, similar changes (440-fold in hypoxia) are obtained in the cells on AC gel substrates as compared with the results in vimentin expression (Figure 7c). For transcription factors ZEB1 (Figure 7e) and SNAI2 (Figure 7f), more upregulation of ZEB1 and SNAI2 in the cells incubated on AC-soft and AC-mid substrates in hypoxia has shown a trend to further induce EMT. The behavior is consistent with the results obtained in TGF-β expression under both normoxic and hypoxic conditions. As expected, the expression level of epithelial cell marker CHD1 associated with EMT is significantly lower than that observed in CDH2 expression level (Figure 7d), indicating that EMT is more promoted in both normoxic and hypoxic environment.

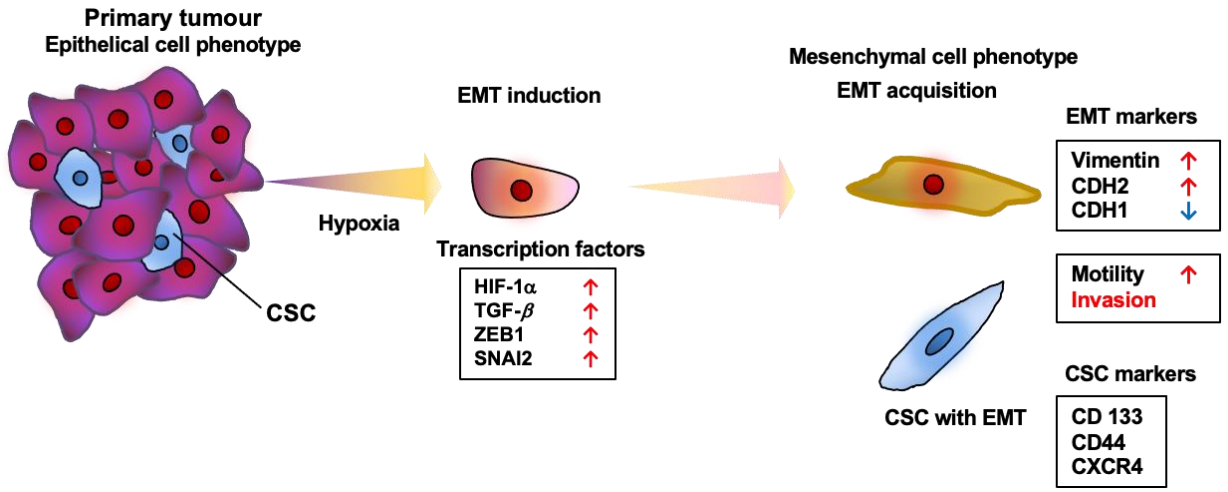

Fig. 6: Transcription factors and relevant markers driving EMT. The figure on left represents cells with an epithelial phenotype while the figure on right represents cells with a mesenchymal phenotype.
The changes in gene expression that contribute to the repression of the epithelial phenotype and activation of the mesenchymal phenotype involve master regulators, including hypoxia inducible factor 1α (HIF-1α), transforming growth factor β (TGF-β), snail family zinc finger 2 (SNAI2), and zinc-finger E-box-binding (ZEB1) transcription factors. Their expression is activated early in EMT.
E-cadherin (CDH1) is the major component of epithelial adherent junctions which mediate, along with tight junctions, intercellular adhesion. The down-regulation of E-cadherin is one of the most significant hallmarks of EMT, which loss of expression can induce the occurrence of EMT.
N-cadherin (CDH2) express in stromal cells, fiber cells, tumor cells and neural tissues, whose levels is closely related to invasion, death and migration. It is well known that cadherins transfer from E-cadherin (CDH1) into N-cadherin (CDH2) during EMT.
Vimentin is a mainly expresser in fibroblasts, endothelial cells and the intermediate filament of matopoietic cells. The expression of vimentin which is related to invasion and metastasis, with the result of acting as a marker of EMT.
In breast cancer, $CD133^+$ and $CD44^+$ cells or cells with high aldehyde dehydrogenase (ALDH) activity have been shown to be enriched in breast cancer stem cells. EMT signaling is involved in development and maintenance of breast CSCs.

Taken together, these results indicate that AC-soft and AC-mid substrates cause significant change in induction (transcription factors (ZEB1 and SNAI2) associated with EMT) and acquisition (vimentin and CDH2 expressions) of the EMT. Up to now, we have limited information regarding viscoelastic gel substrate–mediated EMT[11]. The effect of tanδ feature from AC-soft (tanδ=0.244) to AC-mid (0.193) including AC-s/m (0.201) on gene expression might explain by a further study.

6. Connection between CSCs and EMT

In each of gel substrates tested in this study, the effect of tanδ feature on the gene expression of vimentin and CDH2 is more beneficial for MCF-7 cells under different oxygen concentration levels, in which a significant level of upregulation is evident for the cells incubated on AC-soft substrate.

When we correlate expression of $CD133^-CD44^+$ with vimentin and/or CDH2 in MCF-7 cells, we observe a significant association between $CD133^-$ $CD44^+$ level and vimentin/CDH2 expression in the cells incubated on AC-soft substrate. These results indicate that $CD133^-CD44^+$ level is involved in regulating the expression of HIF-1α. We assume that breast CSCs characterized $CD133^-CD44^+$ level may have a survival advantage under hypoxic condition, since EMT has been demonstrated to contribute to drug resistance in breast cancer[53,54]. So far, the induction of EMT is believed to promote CSC features. CSC theory has been widely accepted as a central principle to explain tumor aggressiveness, recurrence, chemoresistance and even metastasis through EMT phenomenon[9, 53, 55].

The role of CD44, a hyaluronic receptor, is to promote cell-adhesion and assembly of cell surface growth factors, especially in the maintenance of cell-matrix interactions and maintenance of a stem cell phenotype[56]. Mechanisms regarding how tanδ feature controls CD44 expression are obscure in terms of the regulation of a large variety of signaling pathways although main receptor CD44 is identified as main components of CSC niche.

In the clinical setting, studies indicate that circulating tumor cells (CTCs) in patients with metastatic breast cancer frequently express mesenchymal markers, whereas mesenchymal markers are only found in rare cells within the corresponding primary tumours. Notably, it has also been shown that a major proportion of CTCs found in the blood samples of breast cancer patients also express the stem cell marker ALDH[57]. With this in mind, breast cancer cells with or without her EMT during intravascular circulation may induce the CSC characteristics by an induced microenvironment with a very low elastic modulus (~50 Pa) (*cf*. Figure 5b).

For the connection between $CD133^{+}CD44^{-}$ with vimentin and/or CDH2 in the cells, the results in Figure 5a indicate that $CD133^{+}CD44^{-}$ levels are not associated with vimentin/CDH2 expressions (Figure 7c,d). In the $CD133^{+}CD44^{-}$ levels, their tanδ-dependent manner is not contributed by EMT phenomenon and is an independent from acquisition of the EMT. Several studies were reported that CD133 expression contributes to tumor survival under hypoxia. Soeda *et al.*[56] showed that hypoxia could promote $CD133^{+}$ cancer stem-like cells expansion by upregulating HIF-1α. Furthermore, Hashimoto *et al.*[58] also found that hypoxia could encourage CD133 expression with HIF-1α. However, the mechanism of an interaction between CD133 and HIF-1α is still unclear.

In the presented study (Figure 7), they showed the possibility that CD133 does not affects HIF-1α expression as well as vimentin. These findings are at odds with the hypothesis that EMT is necessary to sustain the CSC phenotype, and they imply that the expression between CD133 and CD44 markers are uncoupled each other and CD133 expression does not connect with EMT in each of the substrate conditions tested in this study.

Previous reports showed that high CD133 expression is especially correlated with tumorigenicity, metastasis, and worse prognosis[28,29]. It has a strong potential as a target for drug therapies, since many breast cancer cell lines will express this marker. Indeed, it is still a matter of debate whether $CD133^{+}$cells truly represent the ultimate tumorigenic population. However, the belief that CD133 may act as a universal marker of CSCs has been met with a high degree of controversy in the research community.

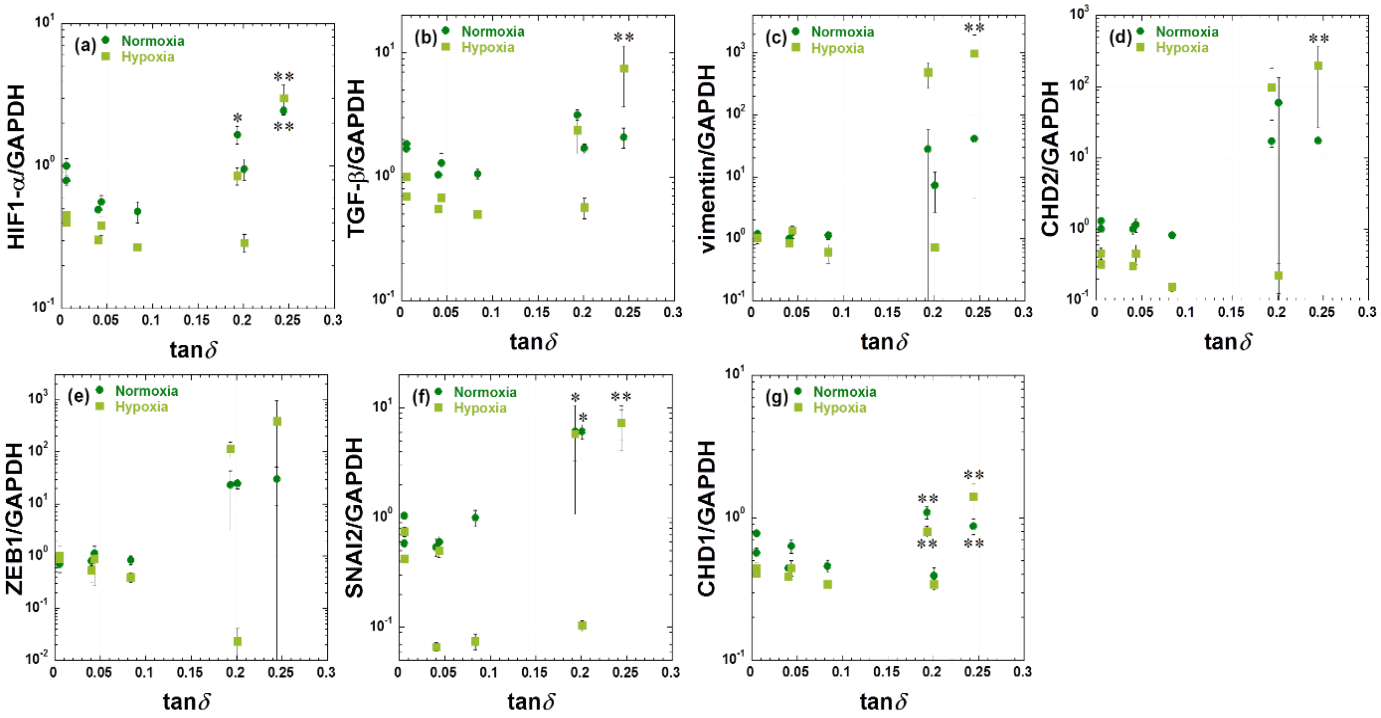

Fig. 7: Effect of the substrate tanδ on gene expression of (a) HIF-1α, (b) TGF-β, (c) vimentin, (d) CDH2, (e) ZEB1 (f) SNAI2, and (g) CDH2 for MCF-7 cells after 7 days culture under both normoxia and hypoxia. (*$p < 0.05$ and **$p < 0.01$). GAPDH, glyceraldehyde-3-phosphatase dehydrogenase. (Copyright 2021. Reproduced from ref. 32).

7. Conclusions and perspectives

The substrate damping as potential physical parameter emerged the important linkage to cellular motility, cancer stemness, and EMT induction. Although further investigation is required to clarify the efficacy of environmental stimuli ($\tan\delta$ feature) for tumors exhibiting CSC-like properties, this report indicates that the MCF-7 cells incubated on softer substrate might lead to express CSC biomarkers exhibiting high CD44 expression.

Thus, targeting the viscoelastic properties of microenvironment around cancer appears to represent a new direction for cancer therapy. To do this, it is necessary to deeply understand not only the cancer cells themself, but also the relationship between cancer cells and their microenvironment with viscoelasticity, including stroma and intravascular circulation.

The role of viscoelastic properties of the microenvironment is just starting to be considered[59-61]. Most important is the recognition that we may have the tool ($\tan\delta$ feature) we need to achieve eradication and cure in the ultimate goal of cancer therapy.

Acknowledgments

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Conflicts of Interests

Author declares that there are no conflicts of interests.

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