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The Effect of Yamanaka Factors on the Metastatic Potential of Breast Cancer Cells

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THE EFFECT OF YAMANAKA FACTORS ON THE METASTATIC POTENTIAL OF BREAST CANCER CELLS

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Abstract

The mortality associated with cancer is most commonly a result of it spreading to far away tissues and organs in a process known as metastasis. For epithelial cancers, such as breast cancers, to do this they must first break and migrate through the basement membrane containing them so that they can interact with the surrounding stroma and spread. This process is known as an epithelial-mesenchymal transition and occurs when carcinomas suppress their original epithelial qualities in order to adopt more mesenchymal ones. This involves cancer cells dedifferentiating from their original cell type to lose the specialized features that distinguished them and where they came from. As cancer cells become more invasive, this dedifferentiation only becomes more pronounced. However, recent breakthroughs in the field of epigenetics have uncovered a way to experimentally reverse dedifferentiation through transgenic expression of a family of transcription factors known as Yamanaka Factors (Oct4, Sox2, Klf4, and Myc). For the purposes of cancer, we hypothesized that this redifferentiation treatment would also reduce metastatic potential. To determine if this was the case, said redifferentiation treatment was conducted on a metastatic breast cancer cell line, 4T1 cells, using liposome transfection. Once the Yamanaka factors were transiently expressed in the 4T1 cells, as confirmed through epifluorescence microscopy of eGFP, measurement of metastatic potential was conducted via flow cytometry of stem cell associated proteins (CD49f and P-Cadherin) as well as a wound healing assay. While there was a significant decrease in CD49f and P-Cadherin levels post transfection, the rate of closure for the wound healing assay was nearly identical. This suggests that while transient expression of Yamanaka factors does alter the differentiation state of cancer cells, it does not alter proliferative and migratory capabilities associated with their malignancy.

Acknowledgements

This paper would not have been possible without the invaluable mentorship and support of Dr. Nicholas Pullen and Dr. James Haughian. My mentor, Dr. Pullen, in particular allowed me the freedom and flexibility to make this project my own despite how it diverged from his own specialization in immunology. He even went so far as to connect me with another professor, Dr. Haughian, whose expertise was more applicable to my working with nucleic acids and bacteria. Regardless, both professors remained critical resources for allowing me to create and complete the project. They not only provided me with their time and insight, but also their facilities and materials, all of which were crucial to the project's success. Additionally, I would like to thank the University of Northern Colorado and the Beta Beta Beta Biology Honors Society for not only providing funding for the project, but also for providing numerous events from which I could present my findings. Lastly, I would like to thank my family for the emotional support they provided at every step. They were instrumental in helping me push through the finish line despite the many difficulties along the way. Thank you to all of you.

Introduction

Cancer is the second leading cause of death in the United States (Murphy et al., 2021). Understanding the mechanisms behind its lethality is crucial to combatting it. Fundamentally, cancers are caused by genetic mutations that dysregulate cell division to constitutively express proto-oncogenes as oncogenes as well as repress or outright delete tumor suppressor genes. For a tumor is but an abnormal overgrowth of cells facilitated by mutations dysregulating the cell cycle to promote rapid and unending cell division. The site containing the initial cancer cells that accumulated enough mutations to drive tumorigenesis is known as the primary tumor. However, only 10% of people with cancer die from their primary tumor. 90% of patients die from cancer cells that have left the primary tumor, traveled through the bloodstream, and colonized in remote organs (Ganesh and Massagué, 2021).

This process of leaving the primary tumor to form secondary tumors at distant sites is known as metastasis. The reason it is so dangerous is because the remote organs cancer cells can spread to after leaving the primary tumor can be much more important to survival than the organ of the primary tumor. For example, in the case of breast cancer, abnormal growth of epithelial cells in the breasts is not typically fatal in and of itself since breasts are not vital to survival. However, breast cancer cells can metastasize to the brain where any dysregulation facilitated by abnormal growth has much more dire consequences considering the importance the brain has for maintaining survival (Sun et al., 2022).

Thankfully, metastasis does not happen instantaneously but is instead a step-by-step process beginning with malignancy. As opposed to a benign tumor, which does not

exhibit particularly aggressive behavior, malignant tumors are those that have achieved local invasion into the stroma where they can interact with crucially important support cells such as blood vessels, adipocytes, nerves, and fibroblasts. In successful cancers, it is the stromal cells that make up the majority of the tumor microenvironment, reaching up to 80% in some cases (Farc and Cristea, 2020). These stromal cells are manipulated to aid the tumor in its growth and spread, thus making them crucial for eventually achieving metastasis.

For epithelial cancers, such as breast cancers, to interact with the stroma they must undergo an epithelial-mesenchymal transition. This involves the cancer cells acquiring the capabilities to begin dissolving the basement membrane with matrix metalloproteinases. From there the cancer cells can migrate through the basement membrane into the stroma to establish survival connections with stromal cells. Such interactions would not normally be possible but as the name epithelial-mesenchymal transition suggests, this transition involves epithelial cells taking on properties more associated with mesenchymal cells. For example, epithelial markers, such as E-cadherin and cytokeratin, are repressed while mesenchymal markers, like PDGF, vimentin, and N-cadherin, are expressed (Naderi et al., 2022). Through this transition the cancer cells alter their cellular identity to be more compatible with the stroma which thereby allows them to better interact with it to facilitate malignancy and spread.

After breaking through the basement membrane to gain access to the stroma, the cancer cells of malignant tumors now have access to the bloodstream where they can circulate and eventually exit particular blood vessels to gain access to other parts of the body. Still, just because the cancer cells have access to these other organs via the bloodstream, that

does not mean they will successfully colonize. Managing to form a secondary tumor in a distant organ is very difficult. For example, a third of breast cancer patients typically have thousands of breast cancer cells circulating in the bloodstream and landing in distant sites, but only half of said patients will suffer any actual metastatic secondary tumor (Braun et al., 2005). Colonization is simply an extremely inefficient process that requires metastatic cancer cells to become highly specialized in order to properly engage with the local stroma (Giancotti, 2013).

It remains difficult to predict which malignant primary tumors will metastasize since different cancers have different propensities to metastasize in particular peripheral organs. The seed and soil hypothesis suggests that malignant cells can only colonize in an organ that provides a hospitable enough environment for survival and proliferation (Ribelles et al., 2013). However, the organs with the most hospitable environments are not particularly intuitive. For example, breast cancers in the left breast very rarely metastasize to the right breast and vice versa.

A potential explanation for what determines the success of metastatic colonization in particular organs as opposed to others is that it is dependent on how the primary tumor has manipulated its cellular identity. Similar to what we see with epithelial-mesenchymal transitions early on in the process of metastasis, whereby epithelial cancer cells take on more mesenchymal like qualities in order to be able to interact with the otherwise foreign stromal cells, a similar thing may be happening with colonization. Thus, the determining factor for if a particular metastatic cancer cell will colonize and form a secondary tumor might be whether or not its cellular identity permits favorable interactions with the foreign tissue it finds itself in.

Labeling differentiation as the potential culprit behind successful metastasis bears similarities with a theory taking form in the field of epigenetics to explain the aging process. Known as the information theory of aging, it suggests that aging is driven by chromatin modifiers being relocalized over time which results in epigenetic dysregulation and thus a loss of cellular identity (Oberdoerffer and Sinclair, 2007). Normally your epigenome is tightly regulated by silencing complexes. As you get older and accumulate DNA damage from replication errors or environmental factors it triggers a redistribution of histone-modifying enzymes. This shifts key silencing factors for heterochromatin over to the sites of DNA damage without properly returning them exactly where they were before. Accumulation of this results in changes in the nuclear architecture and tissue-specific gene expression patterns. Eventually all the epigenetic dysregulation results in cells losing their identity and thereby facilitating the symptoms of aging.

While this theory had previously only been supported by yeast models, as of relatively recently it has been shown to also apply to mice (Yang et al., 2023). Using a double stranded break inducing system that did not cause permanent damage to coding regions of the DNA, researchers were able to accelerate the age of the mice almost 50% faster than the control. Then, by transgenically expressing Yamanaka factors, excluding Myc, they were able to reverse the age of the mice. Their findings suggested that not only is epigenetic dysregulation the primary driver of aging, but cells can have their epigenetic information rejuvenated to regain their cellular identity. The Yamanaka factors responsible for this, Oct4, Sox2, Klf4, and Myc, are typically used for inducing pluripotent stem cells (Velychko et al., 2019). However, by excluding Myc you can

instead only partially revert the cell's identity so as to restore epigenetic information instead of entirely erasing it.

Applying these breakthroughs in the field of epigenetics and aging for the purposes of cancer, transgenic expression of Oct4, Sox2, and Klf4 may also restore the cellular identity of dedifferentiated cancer cells. This in turn may reduce their metastatic potential. To test whether or not this is the case, this paper designed an experiment to compare the metastatic potential of a highly metastatic cancer cell line both with and without transgenic expression of Oct4, Sox2, and Klf4.

Methods

Immortalized Cell Culture: The immortalized cell line chosen to serve as a model to test the Yamanaka factor redifferentiation treatment was the 4T1 cell line. This is because it is a highly metastatic triple negative breast cancer which makes it ideal for modeling metastatic behavior. Throughout the experiment, these cells were cultured at 37° C and 5.0% CO_2 within completed RPMI medium. Additionally, sterile technique was consistently implemented to ensure integrity of single cell culture.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR): To determine which, if any, Yamanaka factors were already expressed in the 4T1 cells, their RNA was isolated using a Direct-zol RNA MiniPrep Plus kit. Immediately following this, the RNA was converted to cDNA via a QuantiTect Reverse Transcription kit. From there, the expression levels of the Yamanaka factors were detected with specifically designed primers using the NCBI Primer-BLAST tool. The four Yamanaka factors, Oct4, Sox2, Klf4, and Myc, all had primers designed for Mus musculus, as did Gapdh since it was to be used as the positive control. To ensure products were their expected band sizes, a 10kb ladder was used for reference. For visualization under UV light, SYBR green staining was employed with gel electrophoresis using a 1% agar gel of 50 mL 1x TAE.

Liposome Transfection: Yamanaka factors were transiently expressed into 4T1 cells via transfection using Invitrogen Lipofectamine 3000. The Yamanaka factors were obtained from a pHAGE2-TetOminiCMB-OSK polycistronic plasmid. This plasmid contained Oct4, Sox2, and Klf4 all in one. It was acquired from AddGene and received as an agar stab. The stab was streaked out onto agar plates containing ampicillin at 100 μg/mL and

was left to incubate for 16 hours at 37°C. After the incubation period, a single colony was selected for inoculation in LB broth also containing ampicillin at 100 μg/mL. This medium was then incubated for 16 hours at 37°C and 250 rpm. After the incubation period, the plasmid DNA was isolated using an E.Z.N.A. Plasmid DNA Maxi kit. The isolated plasmid DNA was then transfected into 4T1 cells using Lipofectamine 3000, with the 4T1 cells being plated at $1x10^5$ onto a 24 well plate to achieve 70-90% confluency. The 4T1 cells remained exposed to Lipofectamine 3000 and OSK isolated DNA for 48 hours before the media was replaced. The same transfection process was repeated using eGFP isolated plasmid DNA. Lastly, a control group that received no transfected DNA or Lipofectamine 3000 was also plated.

Epifluorescence Microscopy: To determine transfection efficiency of the 4T1 cells, they were illuminated under an epifluorescent microscope which was used to toggle between fluorescence plus brightfield and just fluorescence in order to compare the normal cell population with the fluorescent cells transiently expressing eGFP. The visualization of eGFP transfection efficiency allowed for estimation of OSK transfection efficiency.

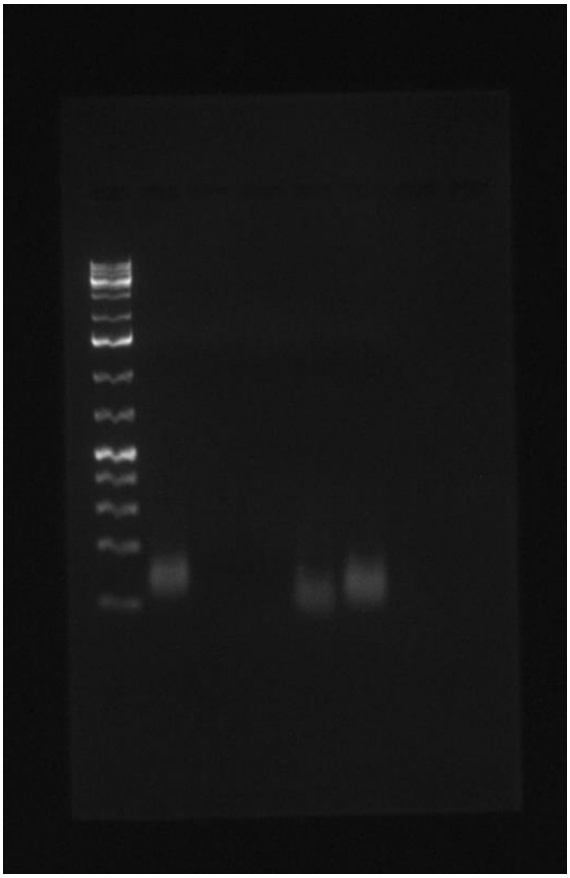
Flow Cytometry: To measure metastatic potential, CD49f and P-Cadherin were chosen as biomarkers to analyze by flow cytometry due to their association with promoting dedifferentiated stem cell like behavior. Similar to the 24 well plated 4T1 cells for liposome transfection, 4T1 cells were plated at $1x10^6$ onto a 6 well plate to achieve 70-90% confluency. The additional size of the plating was done so that there would be enough cells for analysis by flow cytometry. To avoid deterioration of CD49f and P-Cadherin, cells were mechanically scraped instead of trypsinized. Six runs in total were set up, three runs for transfected cells and three runs for control cells. Of the three runs in

each category, one received a CD49f antibody conjugated to FITC, another received a P-Cadherin antibody conjugated to PE, and one received no staining to serve as a control. All runs were washed with 500 µL of PBS after repeated centrifugation. Each run received 2 µL Fc block for 10 minutes as well as 2 µL of their respective stain for 20 minutes under no light. Cells were washed twice more with 500 µL of PBS after repeated centrifugation and from there were resuspended in 1 mL of flow buffer in preparation for flow which had been calibrated to the FITC and PE fluorophores.

Wound Healing Assay: To further measure metastatic potential, transfected cells underwent a scratch assay (wound healing assay) in which a 10 µL pipette tip was scraped along the surface of the adherent 4T1 cells within their 24 well plates. Following the wound creation, snapshots were taken every 2 hours until the wound had fully healed. The rate of closure was analyzed using ImageJ software in conjunction with a plugin specially designed for measuring wound healing assays (Suarez-Arnedo et al., 2020).

Results

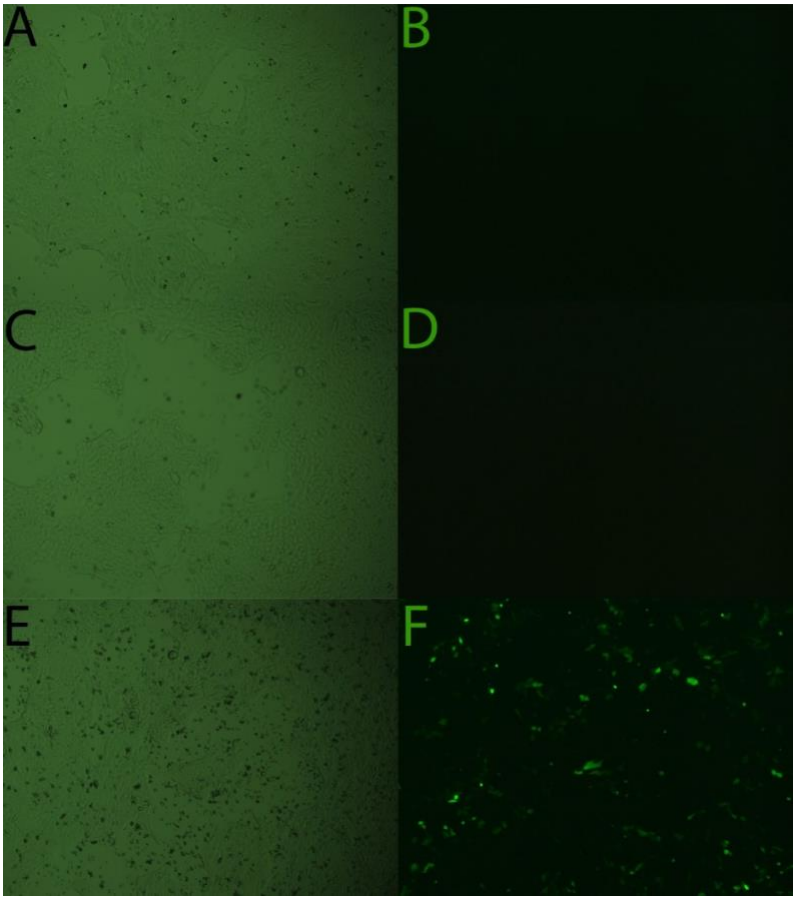
RT-PCR showed that of the Gapdh, Oct4, Sox2, Klf4, and Myc, only Gapdh, Klf4, and Myc were already expressed in the 4T1 cells prior to any transfection (Figure 1). This validates the use of 4T1 cells as the model to test the Yamanaka factor redifferentiation treatment's effect on metastatic potential. Not only were 4T1 cells a highly metastatic triple negative breast cancer ideal for modeling metastatic behavior, but they did not naturally express two of the four Yamanaka factors, Oct4 and Sox2. If they had, another model cell line would have needed to be found since there would be no use transiently expressing genes that are already being expressed. While the 4T1 cells did already express Klf4 and Myc, likely as oncogenes, the lack of Oct4 and Sox2, factors entirely associated with effecting cellular differentiation, is suggestive that their transient expression would still have an impact on the differentiation state of the breast cancer regardless of Klf4 and Myc.



(Figure 1) - Reverse Transcriptase Polymerase Chain Reaction (PCR). Wells from left to right: 10 kb Ladder, Gapdh, Oct4, Sox2, Klf4, and Myc.

Following transfection of the 4T1 cells via Lipofectamine 3000, epifluorescence microscopy showed that the transfection efficiency of the 4T1 cells was ~30% (Figure 2). While 4T1 cells are not optimized for liposomal transfection, as opposed to something like HEK 293 cells, a transfection efficiency of ~30% is still a relatively large amount of transient expression. This is especially the case when we factor in the complete lack of Oct4 and Sox2 in the normal 4T1 cell population. Despite the OSK plasmid DNA lacking a fluorescent marker, assuming it has a similar transfection efficiency to the visualizable eGFP, it will still be a useful way to rejuvenate the cellular identity of the 4T1 cells. This is especially true when we consider the complete lack of autofluorescence occurring in

the nonfluorescent containing wells (Figures 2B and 2D). This indicates that none of the fluorescence seen in eGFP well (Figure 2F) is due to anything but eGFP transient expression.



(Figure 2) - Epifluorescence microscopy.

(A) - Control, fluorescence + brightfield

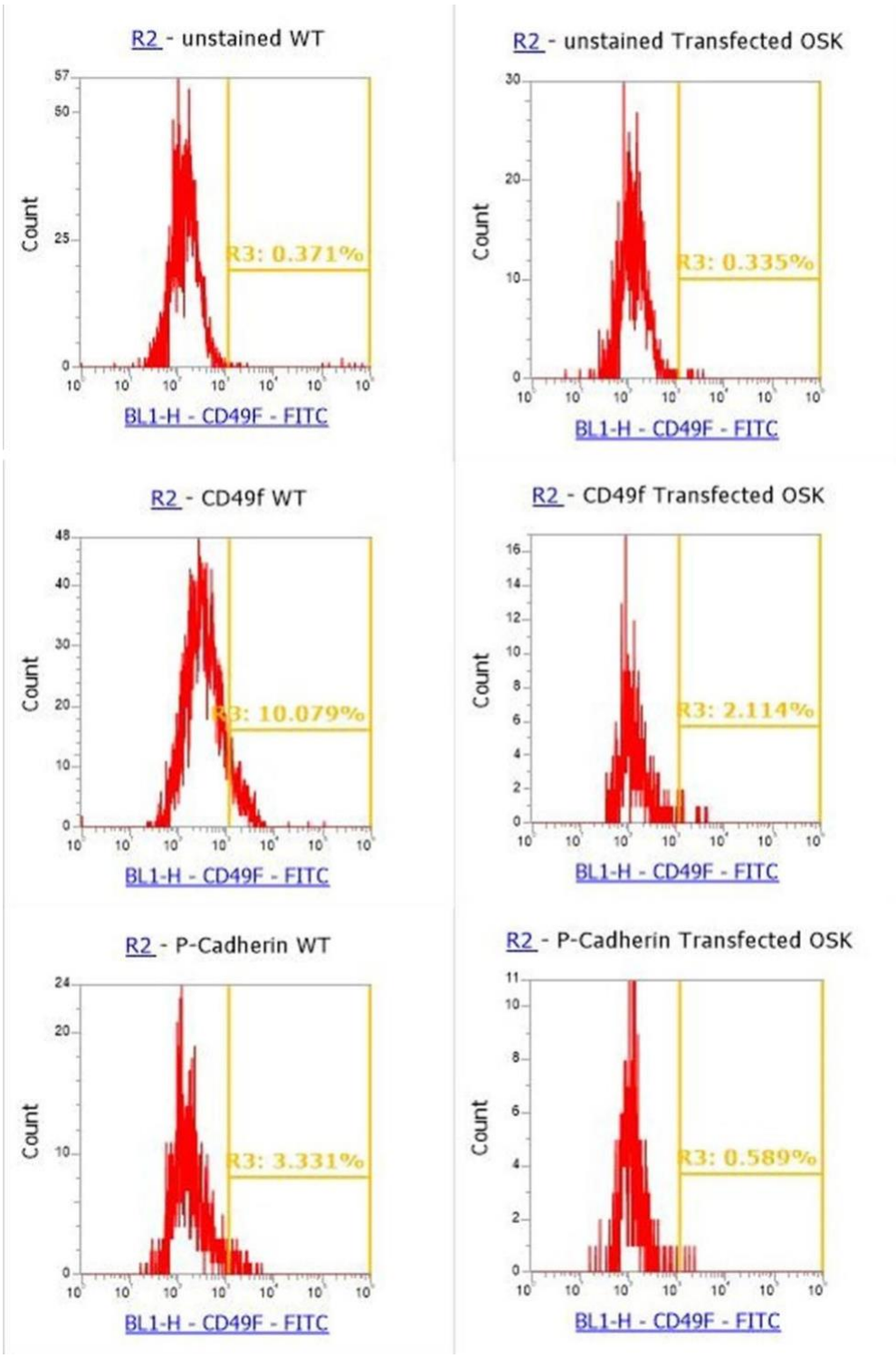
(B) - Control, fluorescence only

(C) - OSK transfection, fluorescence + brightfield

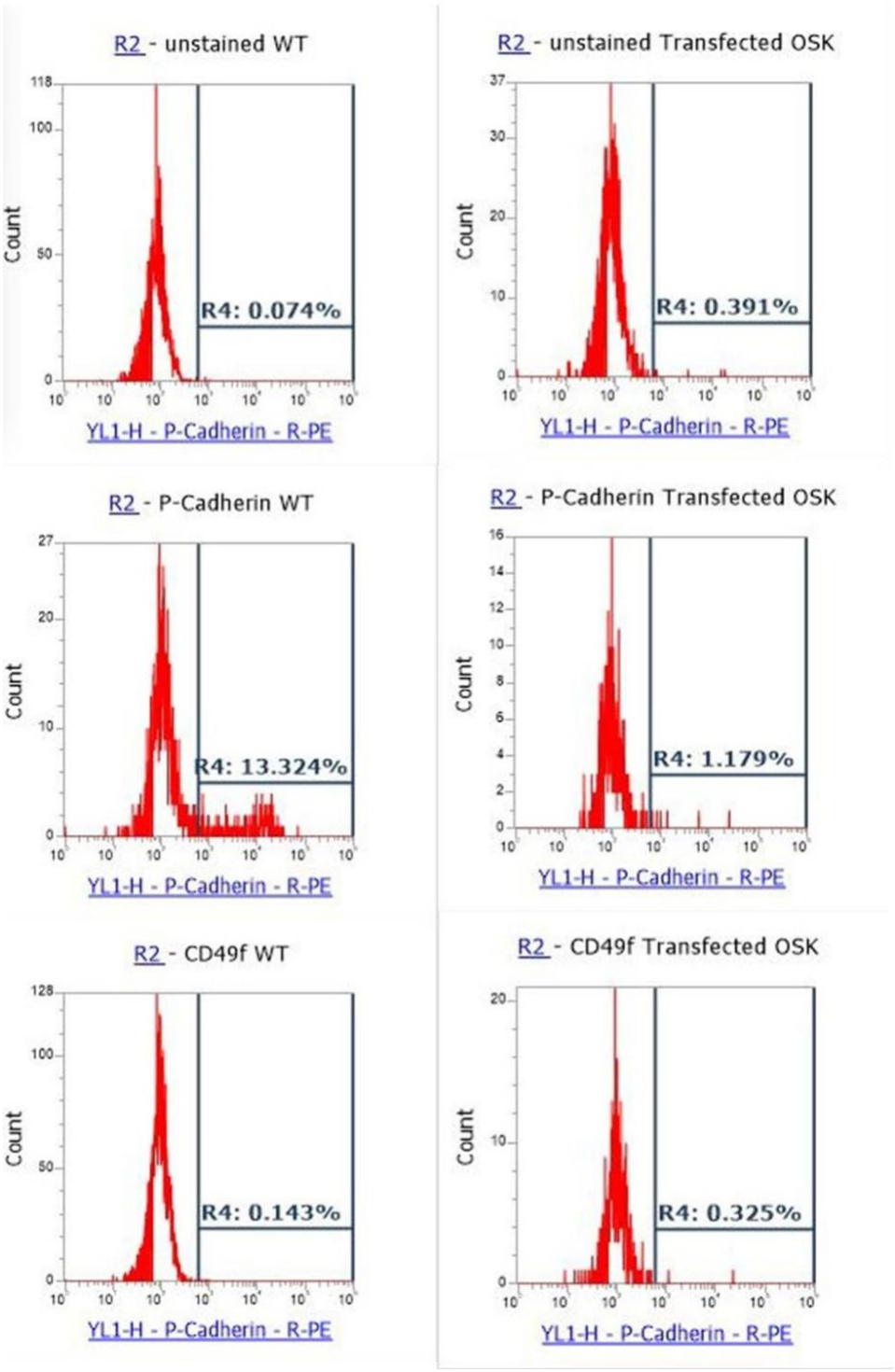
(D) - OSK transfection, fluorescence only

(E) - eGFP transfection, fluorescence + brightfield

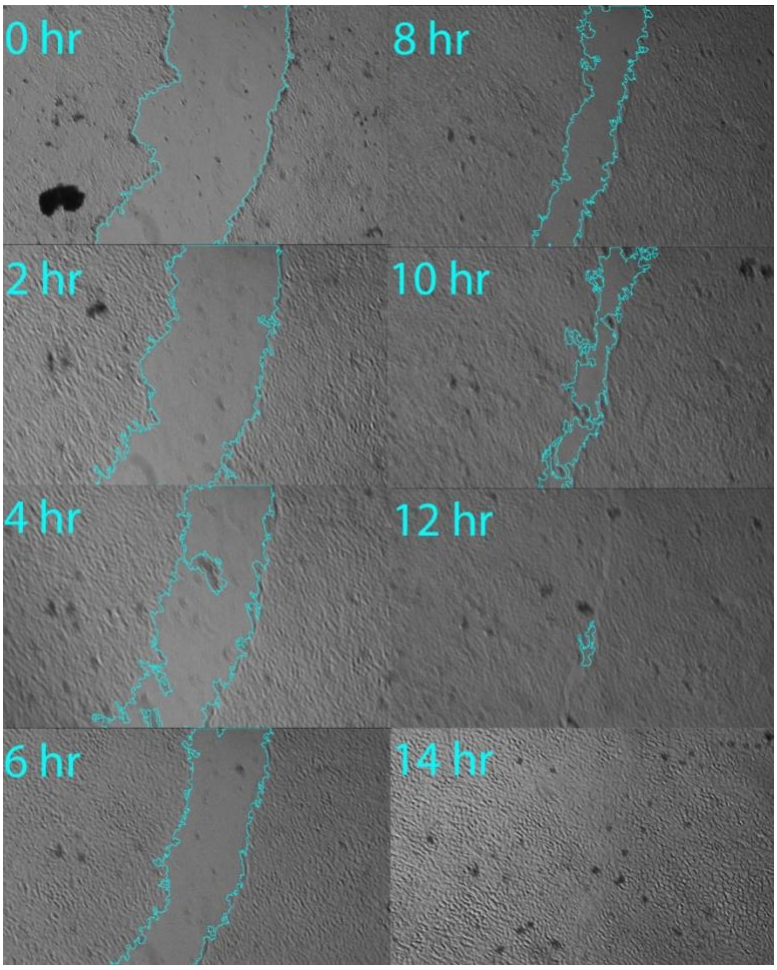
(F) - eGFP transfection, fluorescence only, ~30% transfection efficiency



(Figure 3) - Flow cytometry FITC for all six runs. WT = control. CD49f antibody was the one conjugated with the FITC fluorophore. Decrease in CD49f post OSK transfection, 7.965% reduction.



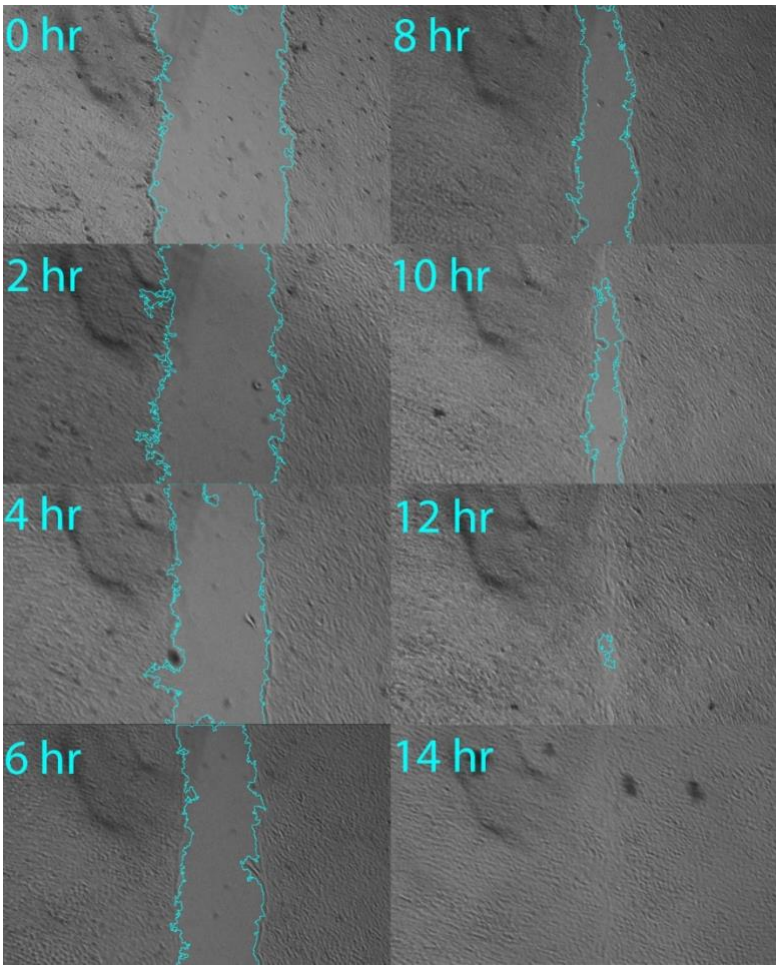
(Figure 4) - Flow cytometry PE for all six runs. WT = control. P-Cadherin antibody was the one conjugated with PE fluorophore. Decrease in P-Cadherin post OSK transfection, 12.145% reduction.



(Figure 5) - Control group wound healing assay over a 14 hr period measured in 2 hr increments. Blue lines outline the perimeter of the scratch.

	Label	Area unit^2	Area %	Width unit	Standard deviation unit
1	0 hr control.jpg	120078.478	30.772	227.496	20.064
2	2 hr control.jpg	105704.702	27.088	208.311	18.989
3	4 hr control.jpg	80204.808	20.497	169.097	17.503
4	6 hr control.jpg	72236.762	18.512	143.088	11.965
5	8 hr control.jpg	44696.780	11.454	91.337	17.605
6	10 hr control.jpg	24528.823	6.286	61.706	21.671
7	12 hr control.jpg	1236.235	0.317	16.828	8.653

(Table 1) - Scratch control, 1 unit = 1 μm



(Figure 6) – OSK transfection group wound healing assay over a 14 hr period measured in 2 hr increments. Blue lines outline the perimeter of the scratch.

	Label	Area unit^2	Area %	Width unit	Standard deviation unit
1	0 hr OSK.jpg	122542.588	31.403	250.630	12.886
2	2 hr OSK.jpg	111981.434	28.697	236.862	24.240
3	4 hr OSK.jpg	87605.498	22.450	183.295	17.992
4	6 hr OSK.jpg	71252.552	18.260	146.473	15.295
5	8 hr OSK.jpg	45959.293	11.778	95.240	16.863
6	10 hr OSK.jpg	20116.597	5.155	48.019	18.009
7	12 hr OSK.jpg	996.155	0.255	15.195	7.522

(Table 2) - Scratch OSK transfection, 1 unit = 1 µm

After transfection efficiency was confirmed, flow cytometry measurements of CD49f and P-Cadherin fluorescence via FITC and PE conjugated fluorophores found that there was a decrease in fluorescence in OSK transfected 4T1 cells from the control 4T1 cells that were not transfected. In the CD49f stained control cells, they recorded 10.079% fluorescence of the FITC conjugated fluorophore (Figure 3). Meanwhile in the CD49f stained OSK transfected cells, they recorded 2.114% fluorescence of the FITC conjugated fluorophore (Figure 3). Similarly, in the P-Cadherin stained control cells, the recorded 13.324% fluorescence of the PE conjugated fluorophore (Figure 4). Meanwhile in the P-Cadherin stained OSK transfected cells, they recorded 1.179% fluorescence of the PE conjugated fluorophore (Figure 4).

These results showcase that through transfection of OSK, 4T1 cells decrease expression of stem cell associated proteins like CD49f and P-Cadherin. This is indicative of the Yamanaka factors altering the cellular identity of the 4T1 cells via epigenetic rejuvenation similar to how they were used to reverse aging symptoms. However, while the cellular identity of the 4T1 cells does seem to be being changed via the transient expression of Oct4, Sox2, and Klf4, the wound healing assays bring into question the relevance that has towards metastatic potential.

Unlike the differences between the OSK transfected 4T1 cells and the control 4T1 cells in the flow measurements for CD49f and P-Cadherin, the wound healing assays showed no difference between the two groups. Both the control and the OSK transfected groups fully healed from being scratched after 14 hrs (Figures 5 and 6). Since the area of the wound started out nearly identical between groups, ~120,078 μm^2 for the control and ~122,543

μm^2 for the OSK transfected, it is not as though one had a faster/slower rate of closure which was masked by a larger/smaller initial wound size (Tables 1 and 2).

This is further confirmed when mathematically establishing the rate of closure using a linear regression line of best fit. In regard to the control group, the rate of closure was 9,270 μm^2/hr at $R^2 = 0.9836$. Meanwhile, in regard to the OSK transfected group, the rate of closure was 9,765 μm^2/hr at $R^2 = 0.9813$. These rates of closure are nearly identical and not indicative of any significant difference induced by the treatment.

Conclusion

While there was a significant decrease in CD49f and P-Cadherin levels post transfection, the rate of closure for the wound healing assay was nearly identical. This suggests that while OSK transient expression alters the differentiation state of cancer cells, it does not alter proliferative and migratory capabilities associated with malignancy. However, that does not necessarily mean that altering cellular identity through epigenetic rejuvenation via OSK transgenic expression has no relation to metastasis at all. Wound healing assays are purely measures of proliferation and migration. While restoring cellular identity may be too little too late in that regard for cancers as aggressive as 4T1 cells, there could still be potential to undermine colonization capabilities. This is difficult to measure in vitro and so future research exploring the effect of Yamanaka factors on metastatic breast cancer may want to utilize an in vivo model to see if secondary tumor formation is reduced post OSK rejuvenation treatment. Another direction to take would be to better classify the exact changes to the cancer's cellular identity. While CD49f and P-Cadherin are indicative of stem cell like behavior, a more comprehensive and sophisticated analysis should be done to ascertain what exact changes the Yamanaka factors are making to the cancer cells.

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Appendix A

Primers used for RT-PCR:

Gapdh F – GGCAAATTCAACGGCACAGT

Gapdh R – GTCTCGCTCCTGGAAGATGG

Oct4 F – GGCTTCAGACTTCGCCTTCT

Oct4 R – TGGAAGCTTAGCCAGGTTCG

Sox2 F – TTTGTCCGAGACCGAGAAGC

Sox2 R – CTCCGGGAAGCGTGTACTTA

Klf4 F – GCACACCTGCGAACTCACAC

Klf R – CCGTCCCAGTCACAGTGGTAA

Myc F – ACCACCAGCAGCGACTCTGA

Myc R – TGCCTCTTCTCCACAGACACC