The Effects and Mechanisms of Lactate and Protons on the Human Umbilical Vein Endothelial (HUVEC) and Murine Melanoma (B16F10) Cells' Migration, Adhesion, and Attachment.

By

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ABSTRACT

The tumor microenvironment is often acidic due to the high metabolic activity of cancer cells, causing the production of excess lactic acid. This elevated acidity significantly impacts the behavior of both normal and cancer cells, including cell migration, attachment, and cell spreading. Under acidic conditions, normal and cancer cells experience delayed migration, attachment, and spreading. In this study, by using cancer cells (B16F10) and endothelial cell (HUVEC) models, it was observed that increased acidity reduced the collective and directional migration compared to treatment at physiological pH, resulting in slower wound closure. A further reduction in collective and directional migration occurred under the combined effect of acidity and elevated lactate concentrations. Furthermore, increased lactate concentrations at the physiological pH (pH 7.4) resulted in a similar reduction in cell migration. Interestingly, increasing the acidity caused opposite cellular responses in both models used in this project regarding random motility. The elevated levels of acidosis caused a significant increase in the random motility of B16F10 cells,

marked by the uncoordinated movements of the migrating cells, which is the opposite of what was observed in HUVECs, where acidosis reduced random motility. Moreover, the random motility decreased in both cell models under the combined effect of the elevated acidity levels and lactate concentrations relative to the treatment with acidity alone.

Furthermore, B16F10 and HUVEC cells adapted a higher polarization and elongated cell morphology (shape) in an acidic microenvironment due to difficulty in tail detachment. The same phenomenon was observed in the cells treated with lactate at a physiological pH. However, in both cell models, the combined effect of acidity and lactate had an insignificant impact on cell length compared to acidic treatment only. Moreover, HUVECs formed multiple leading edges of "Lamellipodia" when exposed to an acidic buffer, while the melanoma cells did not exhibit this trait. Also, the percentage of HUVECs with multiple leading edges decreased under the combined influence of lactate and acidity, aligning closer to the levels seen with physiological pH.

The interconnection between cell attachment, spreading, and cell migration was intensively studied. Cell attachment and spreading are the main primary stages in cell migration. They establish the initial contact and provide a correct mechanical force initiation and cellular signaling for the cell to migrate. In both cell models, the attachment and spreading delay was observed in acidic microenvironment, whereas the addition of lactate to the acidic treatment reversed these effects by reducing the negative impact of acidity in this assay. In this assay context, it was observed in both cell models that acidity blocked the transition of spreading cells to the polarized phase, and the combination effect of acidity and lactate reduced this transition blocking.

Actin fibers play a crucial role in cell migration by providing structural support, enabling force generation, and coordinating signaling pathways. In both cell models, significantly high actin stress fibers were observed in an acidic microenvironment compared to physiological

microenvironment. A similar situation was observed in HUVEC treated with high levels of lactate at physiological microenvironment, but not in B16F10 cells. The combined effect of lactate and acidity has an impact on reducing actin stress fibers in HUVECs, but this effect was not observed in the transformed cells.

Changes in the location of focal adhesions and phosphorylation levels of focal adhesion kinase (FAK) and phosphorylated paxillin (pPAX) may significantly impact cell migration, attachment, and spreading. The acidic microenvironment caused alteration in the location and cluster size of phosphorylated pPAX (pPAX, Y118) and phosphorylated FAK (pFAK, Y397) during migration, attachment, and spreading in both cell models, displaying localization to the spreading cell periphery instead of the spreading cell body where dynamic focal adhesions are located compared to physiological pH-treated cells. Acidic treatment combined with lactate reduced the cluster size of pPAX (Y118) and pFAK (Y397) alongside alteration in their localization compared to acidic treatment without lactate. An additional measure was taken to evaluate the effects of acidic treatments on the phosphorylation levels of paxillin and focal adhesion kinase. The acidic microenvironment caused a decrease in FAK and PAX phosphorylation levels in both cell models and enhancement upon the addition of lactate to the acidic buffer in HUVEC but not the transformed cells.

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DEDICATION

Once, someone said “it takes a village to raise a kid”, and I say: “it takes three kids like mine to push a mother like me to reach the top of the mountain she always dreamed of climbing.”

For all the love and support you showed me, for believing in me, and for all the endless nights you had to sleep on the lab floor so I could finish my work on time.

This dedication is for you, my children: Mustafa, Zainab, and Ibrahim.

Also, they said once: “My sister is my first friend and my second mother.”

To my soulmate sister: Dr. Dalal Bajes Salem

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ACS	American Cancer Society
ATP	Adenosine triphosphate
B16F10	Murine melanoma cell line
CD7	ZEISS Cell discoverer 7
CO_2	Carbon dioxide
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
EDTA	Ethylenediaminetetraacetic acid
EGM-2	Endothelial cell growth medium 2
EPPS	N-(2-hydroxyethyl)- piperazine-N0-3-propanesulfonic acid
F-Actin	Filamentous actin
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GPCR	G-protein Coupled Receptor
GPR4	G-protein coupled receptor 4
GTP	Guanosine triphosphate
HCl	Hydrochloric Acid

HEPES	4-(2-Hydroxyethyl)-1 piperazineethanesulfonic acid
HIF-1α	Hypoxia Inducible Factor 1-alpha
HLa	Lactic Acid
HUVEC	Human umbilical vein endothelial cells
ICC	Immunocytochemistry
IgG	Immunoglobulin G
LDH	Lactate Dehydrogenase
MCT-1	Monocarboxylate transporter 1
MCT-4	Monocarboxylate transporter 4
MES	2-(N-morpholino)ethane sulfonic acid
MLC	Myosin light chain
MMPs	Matrix Metalloproteins
NAD^+	Nicotinamide adenine dinucleotide
NaOH	Sodium Hydroxide
NCI	National Cancer Institution
P	Phosphate Group
PBS	Phosphate buffered saline
pH	Potential of hydrogen
pPAX (Y118)	Phosphorylated paxpaxillin PPAX (Y118)
RIPA	Radioimmunoprecipitation assay buffer
ROCK	Rho-associated Protein Kinase
SDS	Sodium dodecyl sulfate
TME	Tumor microenvironment

VEGF	Vascular endothelial growth factors
Y118	Tyrosine 118
Y397	Tyrosine 397

Chapter 1: Introduction

High lactate levels in the serum and the inability to normalize those levels are correlated with unfavorable outcomes in cancer patient care.[1] Recently, lactate is no longer considered a waste product of anaerobic glycolysis. Studies showed the crucial role of lactate molecules in cell migration, angiogenesis, and cancer cell metastasis.[2,3] In cancer cells, lactate is produced excessively due to high rates of anaerobic and aerobic glycolysis. These high levels of lactate were reported to modulate cell migration and angiogenesis: two important biological processes that affect cancer cell metastasis, however, the mechanism for the effect is not fully known.[4] One study showed the altering effect of lactate on the cell growth morphology, where lactate induced noticeable structural changes in how the cells grow and shape.[5] These cellular morphological changes mean a possible change in the expression and/or activation of other proteins that also play major roles in the migration of both transformed and normal cells, such as Focal Adhesion Kinase (FAK), paxillin (PAX), and F-actin.

1.1 The Chemistry of Lactic Acid

Lactic acid (HLa), with the chemical formula $C_3H_6O_3$, serves as a critical weak organic acid with a pKa of 3.86 in metabolism, especially in anaerobic conditions when oxygen is lacking. In water, it dissociates into lactate ($C_3H_5O_3^-$) and protons (H^+) in a ratio of 1:1 lactate: H^+ stoichiometry through the following equilibrium equation:[6]

$$\mathbf{C_3H_6O_3 \rightleftharpoons C_3H_5O_3^- + H^+}$$

HLa has a low dissociation constant ($K_a = 1.38 \times 10^{-4}$), it dissociates in solution to release a hydrogen ion (H+) and a lactate ion.[7] Because of its incomplete dissociation in solution—that is,

a sizable portion stays undissociated at pKa or below— HLa is categorized as a weak acid. On the other hand, dissociation increases as the pH rises above the pKa, which is characteristic of weak acids. It is not because lactic acid behaves like a strong acid that it is primarily dissociated at physiological pH (6-7.4), but rather because of the high pH in relation to its pKa.[7]

In water, lactic acid, which has a pKa of around 3.86, is not completely dissociated into its conjugate base lactate and protons (H^+). Rather, it only partially dissociates, which means that a substantial amount of the acid will stay in its protonated (undissociated) form in a solution when the pH is near to or lower than its pKa. But when the environment's pH rises above the pKa, the weak acid has a tendency to dissociate more and produce more lactate anion, which is its conjugate base. The Henderson-Hasselbalch equation controls this important relationship.[8]

$$pH = pKa + \log\left(\frac{[A-]}{[HA]}\right)$$

Where pKa represents the pH at which half of the acid is dissociated. In the case of lactic acid, it's when the concentrations of lactic acid (HA) and its conjugate base (lactate) are equal (**Figure 1.1**).[9]

HLa is used as a preservative, a curing agent, and a flavoring agent (E270),[10] a byproduct of fermentation, and is responsible for the sharp taste in products like yogurt,[11] and as a pH regulator in processed food, in pharmaceutical, textile, and cosmetics industry.[12]

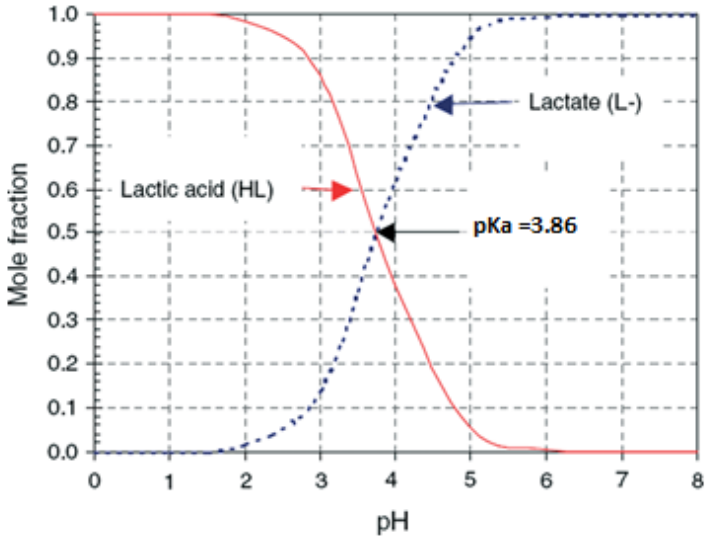


Figure 1.1: Dissociation curve of lactic acid (HLA) at 25 °C with a pKa of 3.86. At pH 7.4, approximately 99.97% or more of lactic acid is dissociated into lactate ($C_3H_5O_3^-$) and protons (H^+), while roughly 99.71% of lactic acid will be dissociated into lactate ($C_3H_5O_3^-$) and protons (H^+) at pH6.4. percentages were calculated using the **Henderson-Hasselbalch equation** in combination with the pKa of the acid. Payan, C., A.-L. Gancel, et al. "Wine acidification methods: a review." OENO One **57**: 113-126

1.2 The Chemistry of Lactic Acid (Lactate and protons) in Biological Systems

HLa is primarily dissociated in biological systems at a pH range between 6-7.4 due to its acid-base properties and the relation between the environment's pH and the acid pKa.[7] As the pH of the biosystem is higher than the pKa of the acid, this causes the environment to have fewer free protons (H^+), which results in shifting the equilibrium towards dissociation to release more protons, which means that lactic acid will mostly be in its dissociated form (lactate) and free protons.[13] Dissociation of lactic acid in biological systems contributes significantly to the body's buffering mechanisms by controlling pH, especially during times of metabolic stress, such as severe exercise or hypoxia. Lactic acid ($C_3H_6O_3$) generated in cells through anaerobic glycolysis dissociates into its conjugate base, lactate ($C_3H_5O_3^-$), and protons (H^+). This dissociation of the HLa raises the concentration of free protons in the intracellular environment causing a reduction in pH. However,

the body's buffering systems, particularly the bicarbonate buffering system, function to neutralize these protons and avoid acidosis. The bicarbonate buffer is determined by the following equilibrium reaction:[7]

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons H_2O + CO_2$$

The protons (H^+) generated through the dissociation of lactic acid are neutralized by bicarbonate ions (HCO_3^-) producing carbonic acid (H_2CO_3). This acid quickly decomposes into water (H_2O) and carbon dioxide (CO_2). Carbon dioxide is later released through the lungs, which helps to eliminate acid from the body and regulate blood pH levels. This buffering mechanism is vital because the accumulation of protons will disturb both intracellular and extracellular pH, resulting in acidosis, which impairs enzyme performance and overall cellular function.[14] Although lactate itself does not directly alter pH levels, it relates to an increase in proton concentration due to 1:1 dissociation ratio for lactic acid which is regulated by the bicarbonate buffering system as mentioned earlier. The muscles release lactate into the blood which is transported to the liver. Via gluconeogenesis, lactate is converted back into glucose and stored in the liver. During this process, protons are used, which serves to balance out any excess acidity that may have developed during the lactic acid generation process.[15]

1.3 The Biological Effects of Lactic acid (Lactate and protons)

Lactate was previously known as a waste product in the cell. Studies showed several biological effects for lactate and protons in the biological system:

1. **Source of energy**: Lactate is a significant source of energy in the cells. When lactate is produced, it can be transferred to other tissues (such as the brain, heart, and liver) where it can

be used as a source of energy by being converted back into pyruvate, this process is called the liver's Cori cycle, which turns lactate into glucose.[16,17]

2. **Signaling molecules**: lactate is considered a signaling molecule in the body that affects many physiological processes, including gene expression alteration, angiogenesis (the formation of new blood vessels), adaptation of muscles during exercises, and immune responses.[13] Lactate's receptors, such as GPR81 (also called hydroxycarboxylic acid receptor 1 or HCAR1), are mostly responsible for the regulation of the signaling function of lactate.[18] GPR81 is a G-protein coupled receptor that is largely expressed in cancer cells and tissues such the brain, muscle, and adipose tissue.[19,20] Lactate's binding to GPR81 triggers intracellular signaling pathways that regulate immune cell activity, anti-inflammatory reactions, and cellular energy balance.[21,22] GPR81 activation is particularly important since it can stimulate angiogenesis, tumor development, and immune evasion, making lactate more than just a metabolic byproduct.[23]
3. **NAD^+ regeneration**: Another biological role for lactate and protons is its role in regenerating NAD^+ During high-intensity exercise. The building up of lactate during the exercise is associated with muscle fatigue, but it also helps regenerate NAD^+. NAD^+ is an essential molecule for continued glycolysis and energy production. [24,25] Studies performed on athletes showed that the responsibility of lactate for muscle soreness is a myth, and it is related more to microscopic muscle damage than lactate accumulation.[26]
4. **Acidification of the Tumor Microenvironment (TME)**: Cancer cells create excess lactate and protons, which are transported into the extracellular space and acidify the TME. This low pH environment promotes multiple cancer-promoting mechanisms, including tumor invasion, immunological suppression, and angiogenesis (the development of new blood vessels).[27] The

acidic TME and poor perfusion[28] can activate proton-sensing GPCRs like GPR4. Activation of GPR4 has been reported to support angiogenic signaling, essential for tumor growth, by influencing endothelial cell function.[29] Additionally, GPR4 and other acid-sensing GPCRs activate signaling pathways leading to the expression of genes associated with inflammation and cancer progression.[30] This suggests that targeting proton-sensing GPCRs like GPR4 is a promising therapeutic approach to eliminate the effects of acidity in the tumor microenvironment, potentially disrupting cancer cell survival and reducing tumor aggressiveness.

5. **Cell Migration and Invasion**: According to some studies, the acidic microenvironment caused by the excess production of lactate and protons enhances cancer cell migration and invasion.[31,32] One of the approaches to this finding is that acidification facilitates the degradation of the extracellular matrix (ECM), making it easier for cancer cells for example to have an efficient metastasis, or break through tissue barriers and spread to distant sites. Lactate can also activate signaling pathways that enhance cytoskeletal rearrangements and cell motility, hence facilitating cancer cell migration.[33]
6. **Immune Suppression**: Lactate and protons inhibit the activity of immune cells in the TME, including T cells and natural killer (NK) cells, which typically target and destroy cancer cells. This permits cancer cells to avoid immune system checks. Acidic environments can reduce immune cell proliferation, cytokine production, and cytotoxic effects.[34]
7. **Promotion of angiogenesis:** Recent studies addressed the indirect role of lactate in promoting the formation of new blood vessels or (angiogenesis) (**Figure 1.2**). This was connected to the role of lactate as a signaling molecule that stimulates the production of vascular endothelial growth factor (VEGF), which leads to the induction and promotion of angiogenesis. The

process is partly driven by the activation of hypoxia-inducible factor 1-alpha (HIF-1α), a key regulator of the cellular response to low oxygen levels associated with the fast proliferation of cancer cells.[35]

8. **Metabolic Flexibility**: Tumor cells shuttle lactate between cells within the tumor. In some types of cancer, cells reconvert lactate back into pyruvate and feed it into their oxidative pathways. This is an adaptation way to enhance energy production while keeping glucose available for other rapidly growing cells in the tumor. This flexibility supports the varied energy and proliferative needs of the tumor (**Figure 1.2**).[36,37,38]

1.4 Cancer and Hallmarks of Cancer

According to the American Cancer Society (ACS), cancer is one of the leading causes of death in the United States. In 2023, 1.9 million new cancer cases are estimated to be diagnosed and about 609,820 cancer-related deaths will occur. Lung, breast, prostate, and colorectal cancer are the most common types of cancers, but skin cancer, particularly melanoma, also presents a significant threat to public health.[39] According to the National Cancer Institution (NCI), cancer is defined as "diseases in which abnormal cells divide without control and can invade nearby tissues, spreading to other parts of the body through the blood and lymph systems".[40] While the definition of cancer according to the NCI is widely used, new approach of more selective definition was adopted, defining cancer as "Cancer is a disease of uncontrolled proliferation by transformed cells subject to evolution by natural selection". [41] Melanoma cancer counts for 1% of all skin cancers, but it's one of the most dangerous types due to its aggressive ability to spread to other parts of the

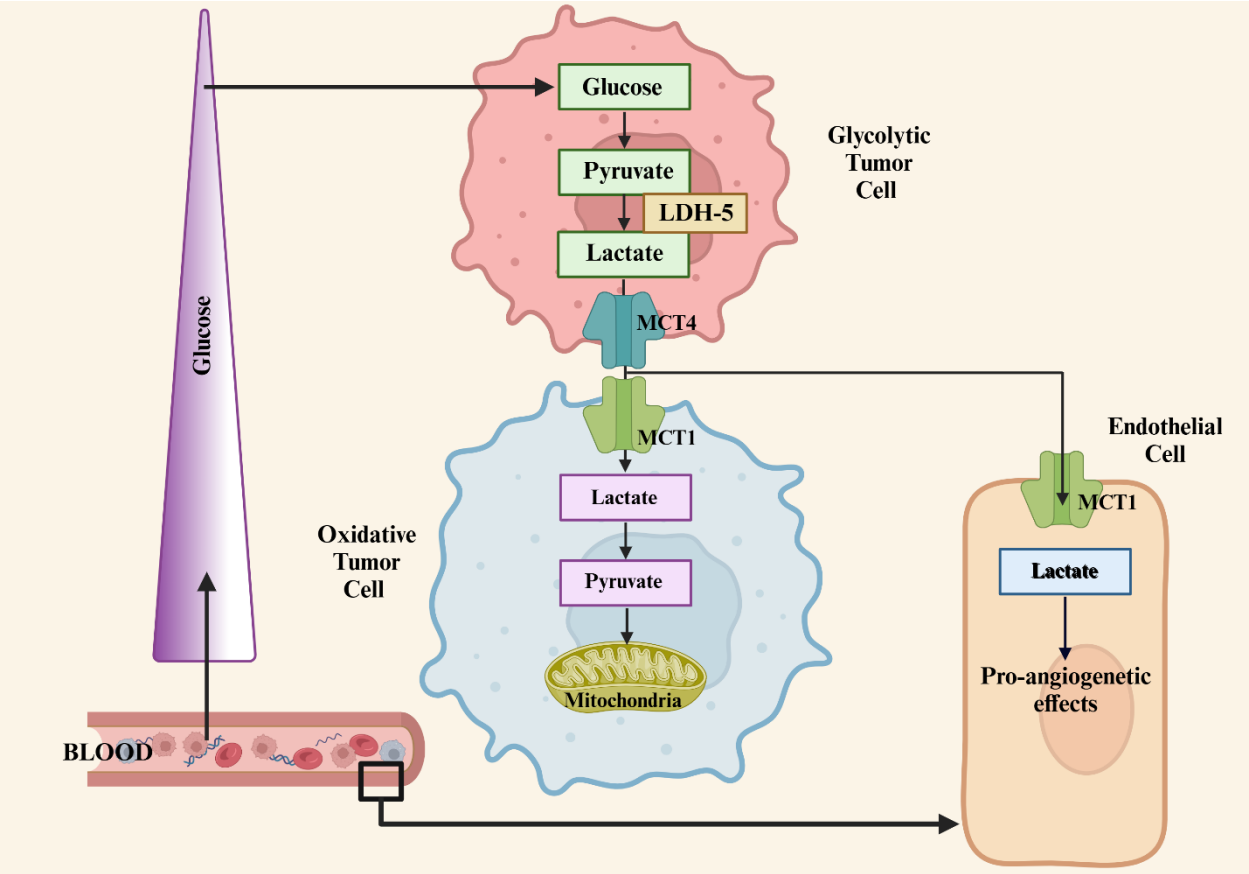


Figure 1.2: Lactate shuttles in cancer cells and endothelial cells. Glycolytic tumor cells produce lactate and release it outside the cell to the TME through a transporter called **MCT4**. Lactate then will be taken through another transporter called MCT1 by either **Oxidative tumor cells or/and Endothelial cells.** Oxidative tumor cells can take in the lactate and convert it back into pyruvate, a key molecule in energy production. This pyruvate can then be used to produce glucose through gluconeogenesis, providing energy for the oxidative cells. Endothelial cells, which line blood vessels, intake the lactate causing the activation of pro-angiogenic pathways that initiate signaling for the growth of new blood vessels or angiogenesis. This graph was created in BioRender. Bajes, F. (2024) BioRender.com/n61y502.

body. According to the (ACS) In 2023, approximately 100,640 new cases of invasive melanoma in the U.S will occur.[39]

The advances in both treatment strategies and the understanding of cancer biology in the recent couple decades caused the cancer survival rates to improve significantly. Jumping from 48% overall “5-year survival rate” for all cancers in 1970s to 69% as of 2023 is a direct reflection of

the more advanced understanding of cancer biology and the implementation of these advances in the continuous development of the traditional treatments such as surgery, chemotherapy and radiation, alongside new strategies such as immunotherapy and targeted therapies.[39,42]

The key for the recent advancements was achievable by a better understanding of the "hallmarks of cancer". In year 2000, Both scientists Douglas Hanahan and Robert Weinberg published a review paper (updated in 2011) defining and concluding the six hallmarks of cancer.[43,44] The hallmarks of cancer are a collection of biological characteristics that identify cancer cells from normal cells, allowing them to develop out of control, bypass the immune system, and spread throughout the body (**Figure 1.3**).[44]

1. **Sustaining proliferative signaling:** proliferation is a vital part of cancer development and progression, it is sustained by the alteration in the expression and/or activity of cell cycle-related proteins in order to continuously signal themselves to grow and divide.[45]
2. **Evading growth suppressors**: cancer cells must avoid signals that exist in the cell to slow down cell growth to bypass the normal regulatory mechanisms that prevent excessive cell growth.[46]
3. **Resisting cell death**: Apoptosis is a method of programmed cell death, it is essential for keeping the tissue homeostasis through preventing the unwanted, surplus, and damaged cells. Cancer cells avoid programmed cell death (apoptosis), allowing them to survive longer through mutations in tumor suppressor genes, overexpression of anti-apoptotic proteins, inhibition of pro-apoptotic signals, or/and inhibition of death receptors.[47,48]

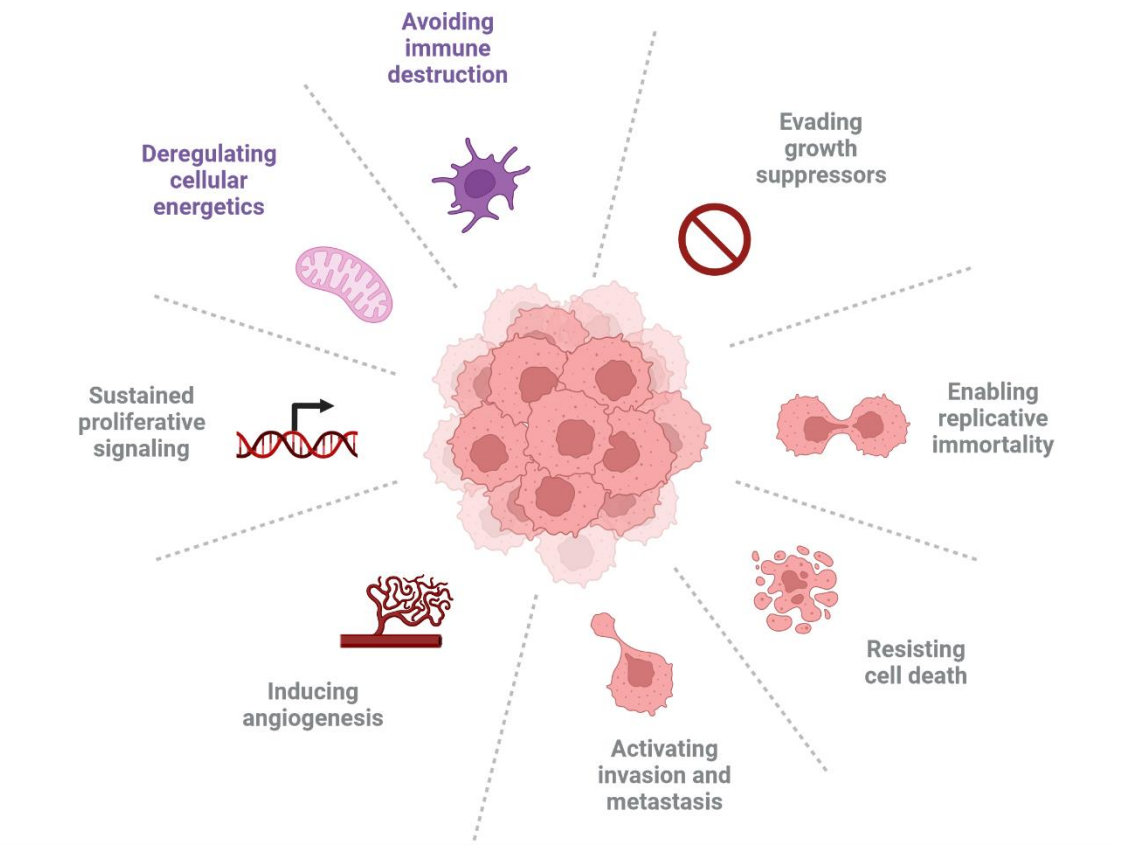


Figure 1.3: Illustration the eight hallmarks for cancer including the two extra added after Douglas Hanahan and Robert Weinberg published a review paper originally proposed in 2000 perspective and the updated perspective in year 2011. This illustration was created in BioRender. Bajes, F. (2024) BioRender.com/l45r551

4. **Enabling replicative immortality**: In contrast to normal cells, cancer cells can multiply endlessly which is known as replicative immortality. This process requires a certain number of mutations in the precancerous cells to overcome the replicative senescence barriers in the cell.[49]

5. **Inducing angiogenesis**: The creation of new blood from pre-existing vasculature is known as angiogenesis. Angiogenesis enables cancer cells to take up nutrients and

oxygen as the original vasculature doesn't provide enough nutrients compared to the fast-growing tumor size. Angiogenesis plays a vital role in the process of the transformation of cancer cells into malignant tumors because solid tumors need a blood supply if they are to grow beyond a few millimeters in size. Angiogenesis, one of the main hallmarks of cancer, has been thoroughly investigated in both human and animal models to create novel anti-cancer therapies.[50]

6. **Activating invasion and metastasis (metastatic dissemination)**: It's a complex process that starts when cancer cells infiltrate the surrounding tissue, move into blood vessels in the area, survive the hostile environment of the bloodstream, and finally leave the bloodstream into distant organs. The cancer cells adjust to their new surroundings once arrive at these new sites. They may start off as undetectable clumps that could eventually develop into tumors.[51]

And in 2011, two emerging hallmarks were added:

7. **Deregulating cellular energetics**: Cancer cells adjust their metabolism to support their growth, survival, and long-term maintenance. High consumption of glucose and the high production of lactic acid (lactate and protons) even with the availability of oxygen, and their mitochondria are operating normally is a crucial part of this change. The metabolism change is referred to as the "Warburg Effect" which is characteristic of cancer metabolism (discussed in the next section).[52]
8. **Avoiding immune destruction**: The immune system's primary function is to defend the body against pathogens including bacteria, fungi, and viruses. It also possesses a number of defense mechanisms to get rid of host cells that exhibit both internal and exterior abnormalities. Cancer cells can create mutations that lead in changing their

characteristics, which causes the cancer cells not to be identified as abnormal cells by the immune system, and thus not get attacked by it.[53]

1.5 Energy Metabolism in Cancer Cells (The Warburg Effect)

Rapidly growing cancer cells and tissues adapt to a lack of nutrients and energy supply through increasing rates of aerobic glycolysis (the Warburg effect). The Warburg effect is a well-known phenomenon that was identified by Otto Warburg, the German biochemist who discovered it in the 1920s. Warburg noted that the energy metabolism of cancer cells is different than that of healthy cells.[52,54] Warburg made an important discovery while looking at how cancer cells use energy. He found that cancer cells consume glucose much more quickly than normal cells and turned most of it into lactate (**Figure 1.4**). This was strange because, usually, cells with healthy mitochondria use oxygen to change glucose into energy through a process called oxidative phosphorylation. This method is more efficient for making energy compared to fermentation, which usually happens when there is not enough oxygen. However, cancer cells preferred fermentation even when oxygen was available causing the production of large amount of lactate. This unusual behavior is known as aerobic glycolysis, or the Warburg Effect [52,55] . The increased absorption of glucose in the Warburg effect is the foundation for diagnostic tools such as ^{18}F-fluorodeoxyglucose positron emission tomography (PET scans) that has become an important tool for detection, staging and management of many types of cancer, which measure glucose metabolism to find tumors in the body.[56]

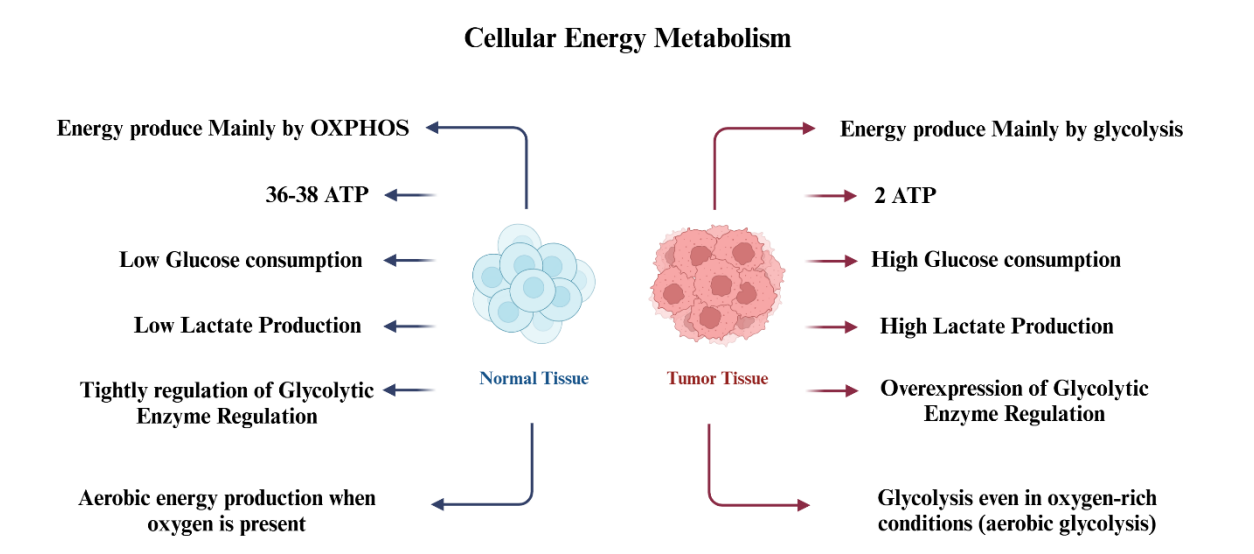


Figure 1.4: Illustration shows how cancer cells modify their metabolism to support rapid growth and proliferation while evading typical cellular energy regulation mechanisms. Created in BioRender. Bajes, F. (2024) BioRender.com/m95g232.

Cancer cells consume more glucose causing high levels of lactate and protons to be produced, leading to creating acidic TME. According to some studies, this metabolic change promotes cancer cell growth by facilitating fast cell proliferation and immune evasion, while weakening immune cells such as T-cells and macrophages in the TME.[57,58] This energy metabolism shift (aerobic glycolysis) produces less ATP per mole of glucose compared to intermediates to feed pathways that produce nucleotides, lipids and proteins that are necessary for OXPHOS but needed by cancer cells for its biosynthetic advantage. The glycolysis generates cell proliferation and growth under the aggressive tumor microenvironment (**Figure 1.5**).[59,60]

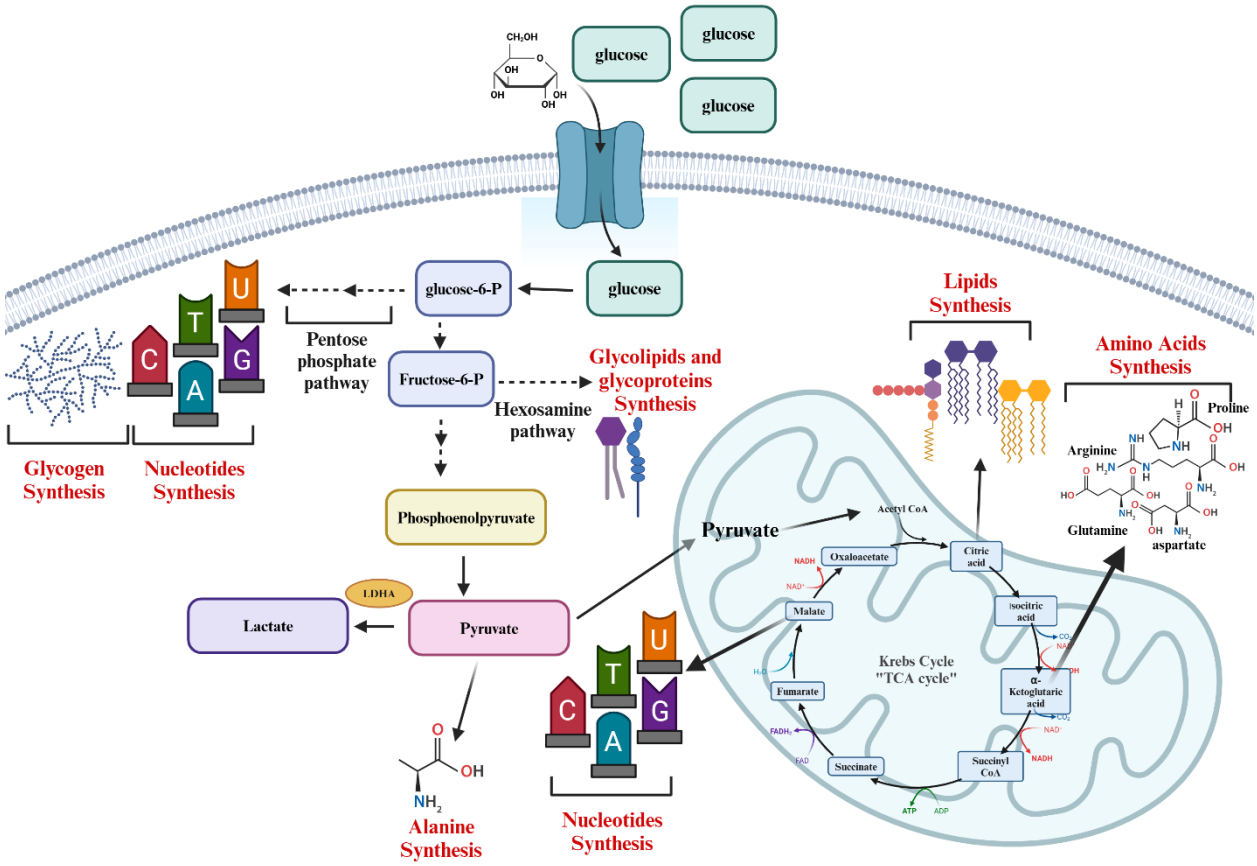


Figure 1.5: Biosynthetic intermediates produced via the shift in the regulation of glucose metabolism in cancer cells. Glucose enters the cell vis one of the GLUT transporter family, then phosphorylated to glucose-6-phosphate (G6P) inside the cell. (G6P) then takes on different fates depending on the metabolic needs of the cell, either get into an oxidative phase and converted after several steps to nucleotides and glycogens via Pentose Phosphate Pathway, or/and further metabolized to fructose-6-phosphate (F6P). F6P will either get into Hexosamine Pathway to produce glycolipids and glycoproteins, or/and get further metabolized to produce pyruvate and ATP. In normal cells (and partially in normoxic tumor cells) Under aerobic conditions, normal cells convert most pyruvate to acetyl-CoA, then oxidized via the TCA cycle, providing sources of ATP synthesis. The metabolic pathways of glucose utilization in glycolytic cancer are changed from ATP generation by oxidative phosphorylation to ATP generation via glycolysis, besides the synthesis of new macromolecules (for example, nucleic acids, lipids, proteins) to facilitate the cell proliferation required for cancer cells to survive the aggressive microenvironment. Created in BioRender. Bajes, F. (2024) BioRender.com/d82e869.

1.6 Warburg Effect and the Production of Lactic Acid (Lactate and Protons)

The high intake of glucose by cancer cells leads to the production of high levels of lactic acid by the enzyme lactate dehydrogenase (LDH), then under the pH of the surrounding it will mostly dissociate into lactate and protons. This reaction includes the conversion of NADH to NAD^+ which means the production of a proton as a byproduct as well. This leads to the accumulation of protons inside the cell.

To prevent acidification inside the cell, monocarboxylate transporters (MCTs) are used by cancer cells to export lactate and protons to the TME (**Figure 1.6**). The lactate and proton accumulation is a major contributing factor to the acidic environment of the tumor microenvironment, this facilitates the rapid generation of ATP and provides the biosynthetic intermediates required for cell's proliferation.[61] Both MCT1 and MCT4 are part of the MCT family, and they play essential roles in cancer metabolism by facilitating lactate exchange, which supports tumor growth and survival. The metabolic demands of cancer cells are fulfilled by the activity by those two transporters especially under the high production of lactate due to the Warburg effect.[62,63]

MCT1 facilitates the transport of lactate into the cell, where it is utilized for oxidative phosphorylation (OXPHOS). In normoxic tumor cells—those located in areas of the tumor with sufficient oxygen—lactate serves as an energy source, thereby preserving glucose for hypoxic cells, which are characterized by low oxygen availability.[64,65] While MCT1 imports lactate into the inside of the cell, MCT4 exports lactate to the outside cellular microenvironment, mainly in glycolytic and hypoxic cancer cells. This process occurs by removing excess lactate and hydrogen targets for anticancer treatments. Inhibiting MCT1 or MCT4 inhibits cancer growth by interrupting lactate exchange, as shown in investigations on leukemia and other malignancies.[66,67]

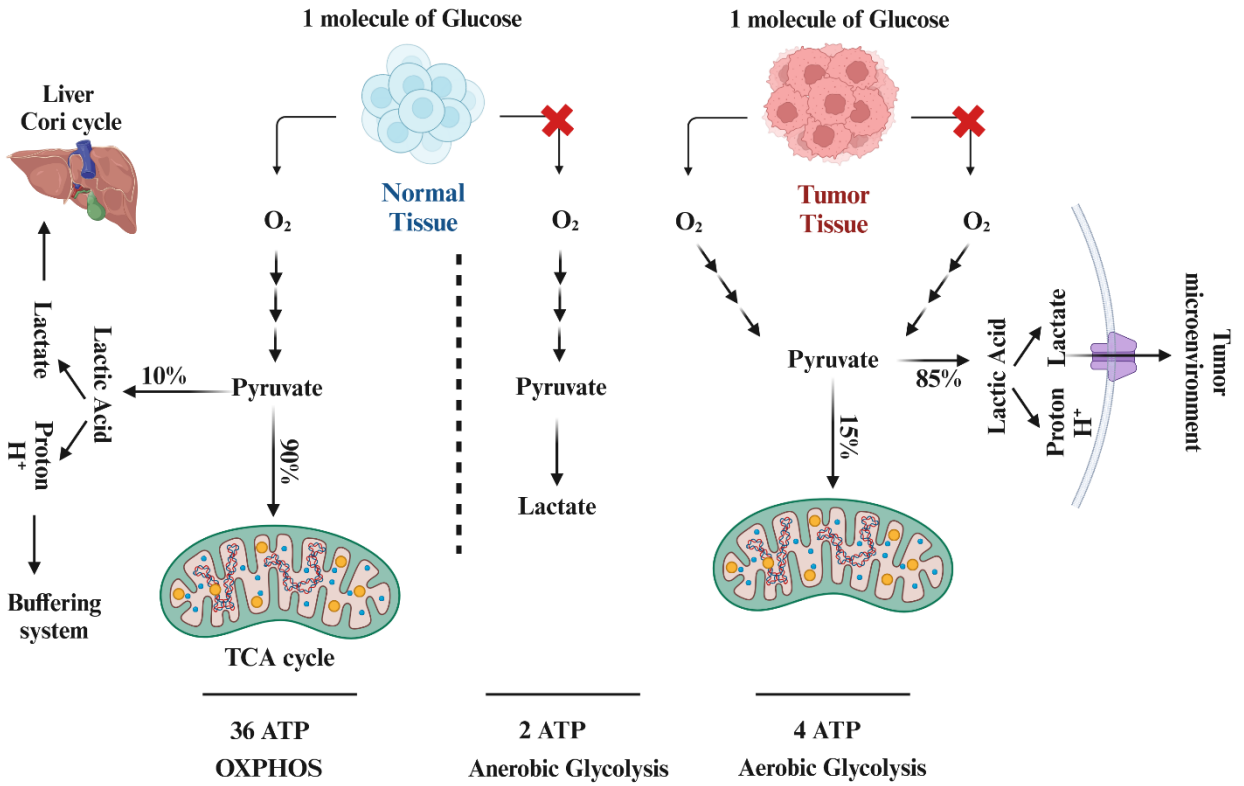


Figure 1.6: A schematic illustration for the metabolic pathway of glucose and the production of lactate in both normal and tumor cells. It shows the differences between OXPHOS, anaerobic glycolysis and aerobic glycolysis, besides the fate of the lactate produced through the three pathways. In normal cells, in the presence of sufficient oxygen, glucose is metabolized to pyruvate that enters the mitochondrial respiration after its transformation. In the anaerobic microenvironment, pyruvate is reduced to lactate in cytosol. 85% pyruvate in malignant cells are fermented into lactate and only 15% pyruvate enter the TCA cycle. mol: molecule. Created in BioRender. Bajes, F. (2024) BioRender.com/c55e894.

When it comes to protons, Cancer cells pump protons (H^+) and lactate out of the cell to maintain their internal environment in a state that supports survival, rapid growth, and metabolic activity. The drop in internal pH damages proteins, DNA, and cellular structures, the internal cellular pH should relatively be neutral or slightly alkaline (around 7.2-7.4) for the cell to function properly. The drop in pH in the cell activates mechanisms to remove excess protons and actively transport them out of the cell to maintain a balanced pH level.[68] MCT1/MCT4 is frequently linked with

aggressive tumor behavior and chemotherapy resistance, making these transporters promising targets for cancer treatment due to their role in facilitating tumor cell metabolism and survival through lactate transport.[69]

Enzymes and signaling pathways associated with growth, including those that regulate protein synthesis and cell division. This environment facilitates the accelerated growth and division of cancer cells, which is essential for the advancement of tumors.[70,71] To maintain the internal pH high, cancer cells pump protons out of the cells to the external microenvironment via transport proteins such as proton pumps (e.g., V-ATPase) and sodium-hydrogen exchangers (NHE1).[72]

1.7 Angiogenesis, Metastasis and Cell Migration

Angiogenesis is a vital biological process by which new blood vessels form from pre-existing ones are created. Angiogenesis is essential for tissue repair and development after injuries, wound healing and reproduction. Endothelial cells (ECs) coordinate this process by migrating, proliferating, and remodeling tissues to create new vascular systems. Cancer cell growth and surviving requires an effective angiogenesis under the acidic microenvironment of the tumor,

where tumors need blood vessel formation to acquire more oxygen and nutrients for fast expansion (**Figure 1.7**).[73,74]

In healthy normal tissues, angiogenesis is a well-regulated biological process that is controlled by a series of signaling and regulating proteins in response to physiological signals such as injury and hypoxia.

First, endothelial cells lining existing blood vessels get activated as a response to pro-angiogenic signals such as vascular endothelial growth factor (VEGF) which is produced after a hypoxia or tissue injury occurs. This activation step results in the release of enzymes like matrix metalloproteins (MMPs) which causes the surrounding basement membrane to be degraded to

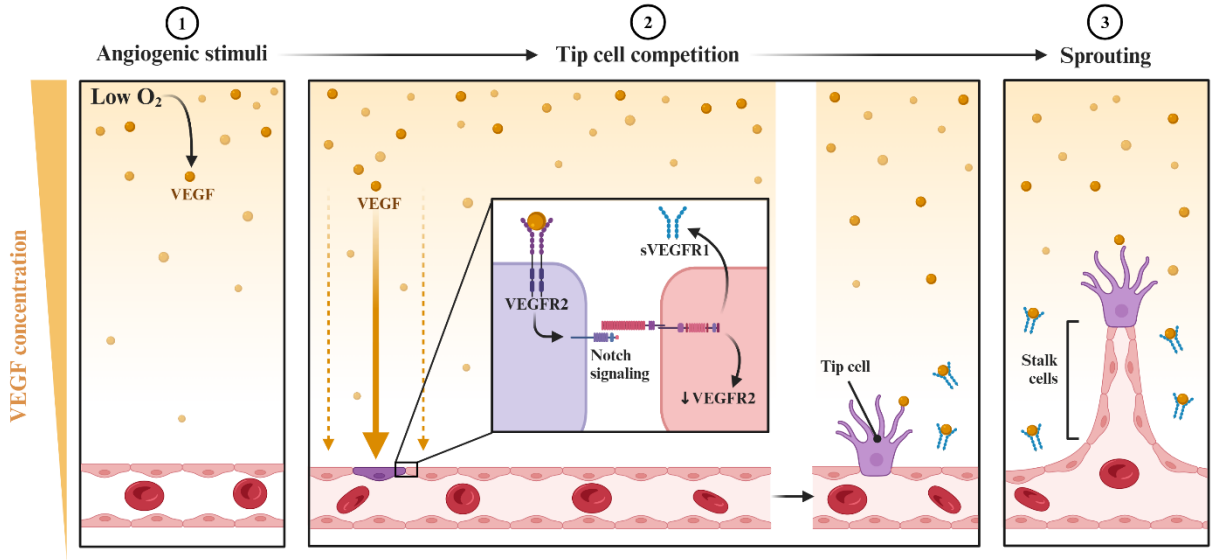


Figure 1.7: Steps of angiogenesis in normal healthy tissues. This figure illustrates the sequential steps of angiogenesis. Triggered by hypoxia or injury, angiogenesis starts with Endothelial cells activation by VEGF, followed by the degradation of the basement membrane, then EC proliferation and migration towards the angiogenic signal forming sprouts from the parent vessel. The migrating cells rearrange to form a lumen within the sprout ending up building a network of connected vessels. The Illustration is created in BioRender. Bajes, F. (2024) BioRender.com/y90q741

facilitate cell migration by creating an open path for cells. After the activation step, the endothelial cells respond to the angiogenic signals by proliferating and migrating, which initiate the formation

of sprouts extended from the parent vessel. The cell migration in this step is led by the "Tip cell" that guides the direction of the vessel growth, alongside the stalk cells that proliferate to extend the sprout. Next, a lumen within the sprout is created by the migrating endothelial cells, and when multiple sprouts meet, branches are formed creating a network of interconnected vessels. Finally, and based on the tissue needs, vessel network remodeling is needed, including regress for the no longer needed vessels, or strengthening to ones needed for long-term blood flow.[73,74,75]

While angiogenesis is a beneficial and regulated process in normal tissues, in tumors it is utilized to fulfill the fast and increased needs and demands for oxygen and nutrients needed for tumor growth and metastasis _ the spreading of cancer cells from the primary site to distant sites in the body _ makes it a hallmark of malignancy (**Figure 1.8**).[76] One of the major differences between angiogenesis in healthy and transformed tissues is the overexpression of pro-angiogenic factors, especially VEGF alongside the downregulation of factors that inhibit the process such as thrombospondin-1. This causes a continuation of forming new vessels without the control to inhibit the switching-off.[77,78] Comparing the vessels of the healthy and tumor tissues, blood vessels in the latter tend to have heterogeneous or irregular organization, function and structure, that means the blood flow will be uneven and have poor delivery of oxygen and therapeutic drugs, leading to resistance to anti-cancer treatments such as chemotherapy.[79,80] Lastly, unlike normal tissues where hypoxia-induced angiogenesis resolves as oxygen levels are restored, cancer cells maintain this process by stabilizing the hypoxia-inducible factor 1-alpha (HIF-1α) _a protein that upregulates VEGF production_ which causes a further promoting angiogenesis [81].

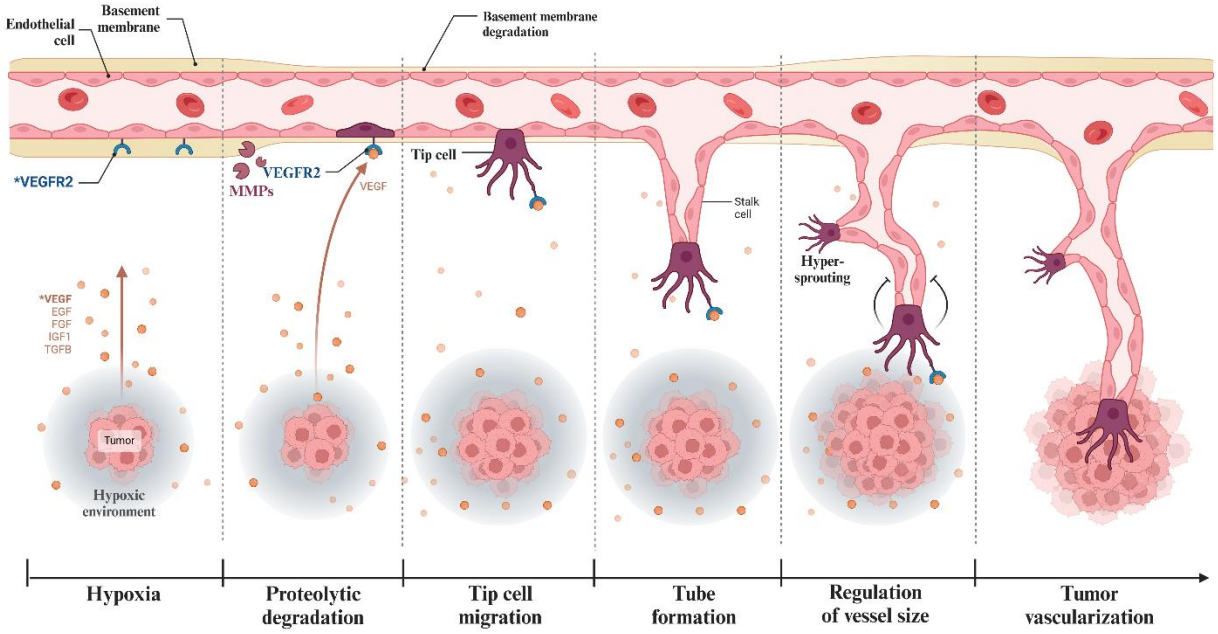


Figure 1.8: Steps of angiogenesis in tumor tissues. This figure illustrates the sequential steps of angiogenesis. Triggered by hypoxia. Angiogenesis starts with Endothelial cells activation by VEGF, followed by the degradation of the basement membrane, then EC proliferation and migration towards the angiogenic signal forming sprouts from the parent vessel. The migrating cells rearrange to form a lumen within the sprout ending up building a network of connected vessels. Tumor tissue has uncontrolled angiogenesis, with uneven, leaky, and disorganized blood vessels growing randomly. This aberrant vasculature permits the tumor to continue its fast development and creates paths for cancer cells to move to other areas of the body (metastasis). The Illustration is created in BioRender. Bajes, F. (2024) BioRender.com/s70x458.

The cells' movement from one location to another is cell migration, and it's a basic biological process that is vital for many physiological and pathological processes[82]. It is necessary for immune responses,[83] tissue repair wound healing,[84] and embryonic development.[85] By directing cells to their correct locations during embryogenesis cell migration enables the correct formation of tissues and organs. In terms of immune response, it makes it possible for immune cells to reach infection or damage sites guaranteeing that the body can react to pathogens or damage efficiently.[83] Cell migration, however, can be dysregulated and lead to the advancement of a disease. One example of this is metastasis in cancer where cancerous cells spread from the original tumor site to form new growths in distant organs.[86] Cell migration is a fundamental step in angiogenesis, even if the regulation and outcomes differ significantly.

The endothelial cells lined in the inner walls of blood vessels migrate toward areas where new blood vessels are needed stimulated by growth factors (VEGF). These factors direct the endothelial cells along a concentration gradient towards hypoxic (low oxygen) regions that require blood vessels as a source of oxygen.[73,74] In transformed tissues, cell migration as well as angiogenesis are dysregulated to match the demand of fast growth and spreading of cancer cells. Transformed cells produce large amounts of VEGF besides other growth factors that induce continuous uncontrolled endothelial cell migration. This dysregulation leads to the formation of irregular and leaky blood vessels that allows cancer to access the circulatory system more easily to serve the effective metastasis of the tumor.[87]

Migration in cancer cells is essential to its development, specifically during the invasion and metastasis phases of the tumor. Cancer cells develop the ability to migrate and enter blood or lymphatic vessels to a new site away from the main tumor site, where they can grow into more tumors. Furthermore, cancer cells that migrate interact with surrounding cells and the ECM to adapt to new environments and survive outside of their original site (**Figure 1.9**).[88] Studying and understanding cancer cell migration is necessary because tumor cell migration plays a key role in the aggressiveness of tumors and their resistance to treatment. This makes it a target for the development of novel therapeutic approaches aimed at preventing the metastases of tumors and enhancing patient outcomes.[89,90]

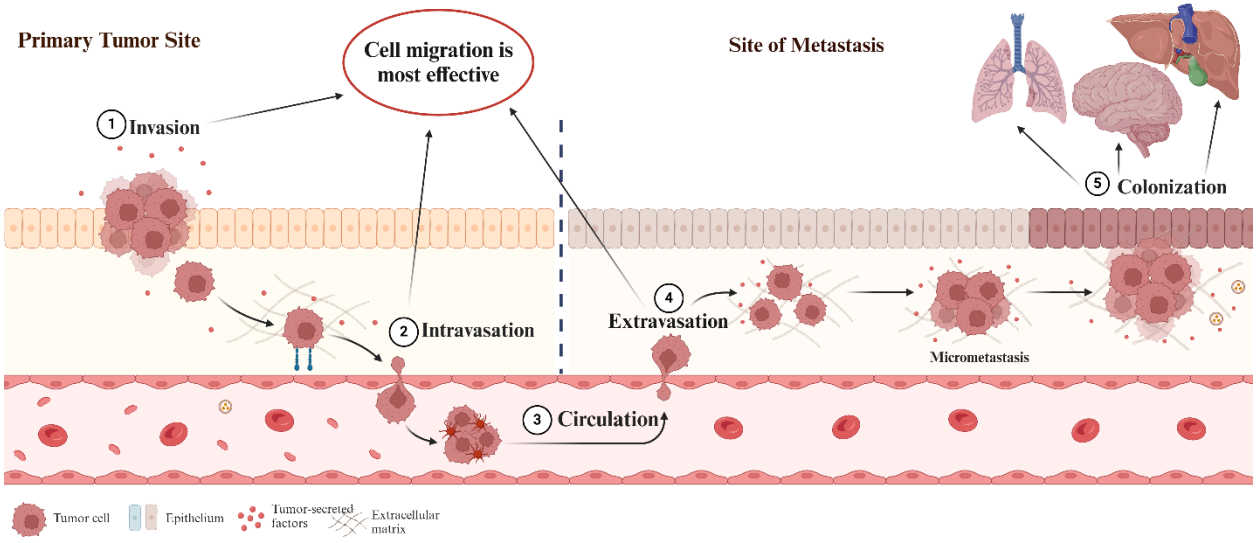


Figure 1.9: Overview of the importance of tumor cell migration in metastatic and invasion cascade. The five key steps of metastasis include invasion, intravasation, circulation, extravasation, and colonization. Cell migration is most effective during the invasion (cancer cells breaking through the basement membrane), intravasation (cancer cells migrate towards and enter blood or lymphatic vessels) and extravasation stages of metastasis (migrating out of the bloodstream). The Illustration is created in BioRender. Bajes, F. (2024) BioRender.com/u46b569.

1.8 Cell Migration: The Multi-Step Biological Process Regulated by Signaling Proteins

The cell migration is a highly complex biological process that involves a group of biochemical signals, coordinated cytoskeletal dynamics, and cellular interactions with the surrounding extracellular matrix.[91] The signaling molecules involved in the cell migration including growth factors, chemokines, and adhesion molecules, guide cells through directional signals to maintain a highly regulated process. The cytoskeleton, mainly composed of actin filaments, provides the necessary structural framework and force for movement via assembling and disassembling. Additionally, cells must continuously remodel their shape, adhere to and detach from surfaces, and navigate through varying tissue stiffness and density. This complex arrangement of events makes cell migration not only a vital component of normal biological

function but also a complex process controlled and regulated by multiple internal and external factors.[92,93,94]

In this project, the focus is on two key proteins, focal adhesion kinase (FAK) and paxillin, which play essential roles in regulating cell migration, attachment, and signal transduction at focal adhesion sites. Focal adhesions are protein complexes that form at the attachment sites between the cell and the extracellular matrix (ECM). These sites act as connection points, linking the cell's internal cytoskeleton to its external environment, enabling the cell to respond to mechanical signals.[95]

The first observable adhesions are called “focal complexes”, they mainly form at the leading edge of the migrating cell. These complexes are “integrin-mediated” adhesions, they contain many proteins that are able to regulate the ECM rigidity of the substrate and the microenvironment including the surrounding cells, and function as Mechan transducers. When the integrin molecules sense the ECM (which acts as a protein binding to the receptor on the cell) the focal complexes are initiated leading to a cascade of protein-protein interactions that include binding of proteins such as talin, vinculin, paxillin, and focal adhesion kinase. Focal complexes are small, highly dynamic, and short-lived, they are directly correlated with cell migration.[96,97]

When the focal complexes mature (or continue growing) into more stable focal adhesions by interacting with additional intracellular proteins such as zyxin through the phosphorylation of those proteins, and with the associating of additional filamentous actin, it is then called focal adhesion. The physical force on focal complexes created by actin polymerization causes the activation of proteins in the cascade and thus causing the initiation of cell attachment to the

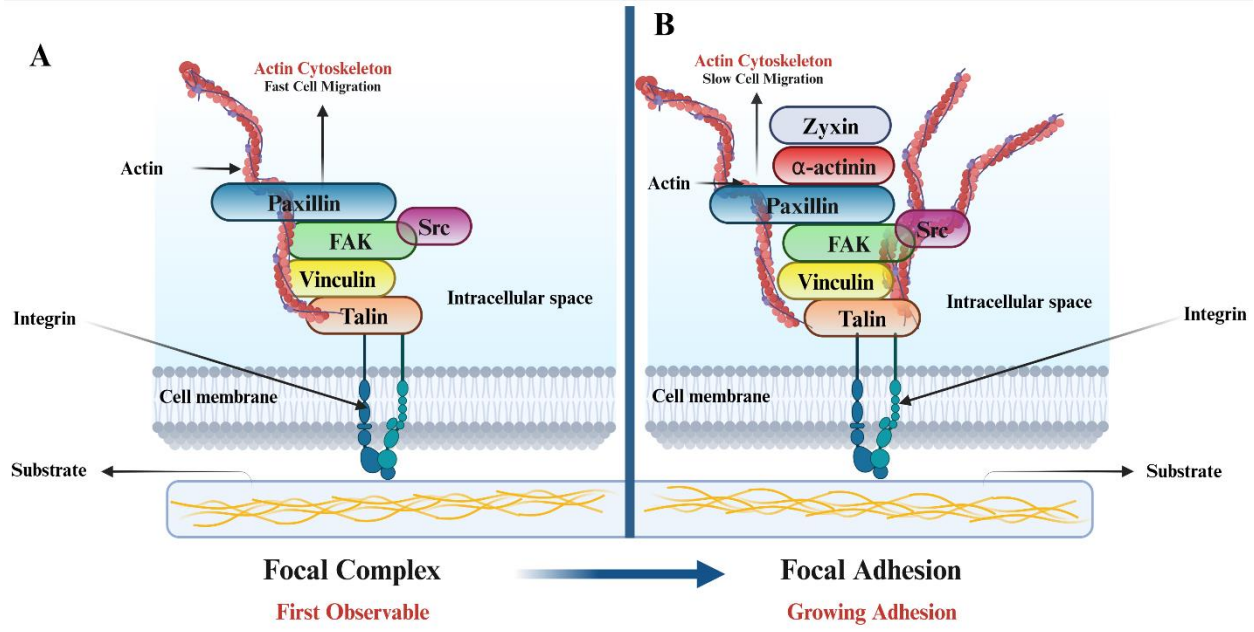


Figure 1.10: Illustration of difference between focal complexes and focal adhesion in a migrating cell. (A) The first detectable adhesions are called focal complexes (B) focal adhesion is a progressively growing in size focal complex by associating with other proteins. Cell adhesions are often initiated by interaction of integrins with their substrates (ECM). The Illustration is created in BioRender. Bajes, F. (2024) BioRender.com/o51d689, and adopted from: Joo, E. E., & Yamada, K. M. (2015). Cell adhesion and movement. In A. Vishwakarma, P. Sharpe, S. Shi, & M. Ramalingam (Eds.), *Stem cell biology and tissue engineering in dental sciences* (pp. 61–72). Academic Press.

substrate and cell migration. Focal adhesions are larger in size and more stable than focal complexes, and they are related to the slower cell migration (**Figure 1.10**).[98,99]

Focal adhesion kinase (FAK) and paxillin are two major proteins that together form and regulate focal adhesions in order to control a well-regulated cell migration through proper communication with the external environment of the cell.

FAK known as "**The Signaling Hub** "is a non-receptor tyrosine kinase, which means it's a protein that depends on the intracellular signals originating from extracellular receptors. FAK plays a major role within the focal adhesions, it is recruited to other focal adhesions that interact

with integrin-linked proteins such as paxillin, and it gets activated through autophosphorylation at tyrosine residue (Tyr397), which creates a high-affinity binding site for other proteins involved in the signaling cascade like **Src** (another kinase). Once active, FAK orchestrates a variety of downstream signals, many of which are involved in controlling cell movement, shape, and survival.[100]

Paxillin, on the other hand, is known as the "scaffolding protein" in the focal adhesions, it binds to multiple proteins involved in cytoskeletal dynamics, including actin that forms the cell's structural framework and signaling molecules like FAK.[101,102] Since FAK is a kinase, its main function is to add a phosphorylation group to specific amino acids (such as tyrosine) in other targeted proteins, in this case FAK phosphorylates paxillin, the scaffolding protein that needs a kinase to be phosphorylated and thus activated [101]. Paxillin contains multiple **LD motifs (**short amino acid sequences composed of **leucine (L)** and **aspartic acid (D)**) and tyrosine phosphorylation sites that can bind to FAK, causing the FAK to localize to focal adhesions, where signaling cascade is initiated. successively, FAK can transform the phosphorylation state of paxillin, regulating its activity and interactions with other proteins.[103]

Based on the physical and functional relation of FAK and paxillin, both proteins work to regulate the cell migration, this occurs by initiating the signaling pathways that regulate the assembly (at the leading edge of the cell) and disassembly (at the rear end of the cell) of focal adhesions which is a vital process required for cell movement. This high level of control of the dynamic turnover of focal adhesions allows cells to "crawl" forward. One important aspect to mention about the FAK and paxillin, is that the phosphorylation acts as a signal for the recruitment of additional proteins that regulate cell motility and survival, their interaction is two-way action. When paxillin is phosphorylated, it can recruit other proteins, such as those involved in **Rho**

GTPase signaling, which further influence the organization of the cytoskeleton and cell movement.[104,105]

In cancer, the FAK-paxillin relationship is often dysregulated, thus this relation plays a significant role in promoting tumor growth, metastasis, and resistance to anti-cancer therapy. Both FAK and paxillin are overexpressed in various cancers, causing great change upon processes such as cell adhesion, migration, invasion, and survival. This overexpression means signaling over activity leading to uncontrolled cell migration, invasion, and metastasis. For this reason, both FAK and paxillin are considered potential therapeutic targets in cancer treatment.[106]

The following is an overview of the main steps involved in cell migration (**Figure 1.11**):

1- **Sensing, the detection of external signals**:

When cells detect external signals, they use specialized receptors such as G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) to initiate the migration process. External signals can be of different types including but not limited to chemical signals (chemotaxis). Electrical signals (electrotaxis) and mechanical signals (durotaxis).[107,108] The sensing step is directed by signaling proteins to ensure the accurate migration process, including:

A- **Rho family GTPases**: Proteins like Rac1, RhoA, and Cdc42 are crucial for sensing and transducing signals that influence the cytoskeleton.[109]

B- **Phosphoinositide 3-kinase (PI3K)** regulate the synthesis of PIP3, a lipid molecule found in cell membranes that is essential for actin polymerization and directs the cell toward stimuli.[110]

C- **Focal adhesion kinase (FAK)**: FAK play a critical role during the cell response to an external stimulation _mainly mechanical forces_. During stimulation,

FAK get activated (FAK auto phosphorylated at specific tyrosine residue (Y397)), which assist the cell to interpret the directional cues, regulating the polarization of the cell in the direction of migration and cell adhesion after provoking multiple signaling pathways related to the cellular events.[111,112]

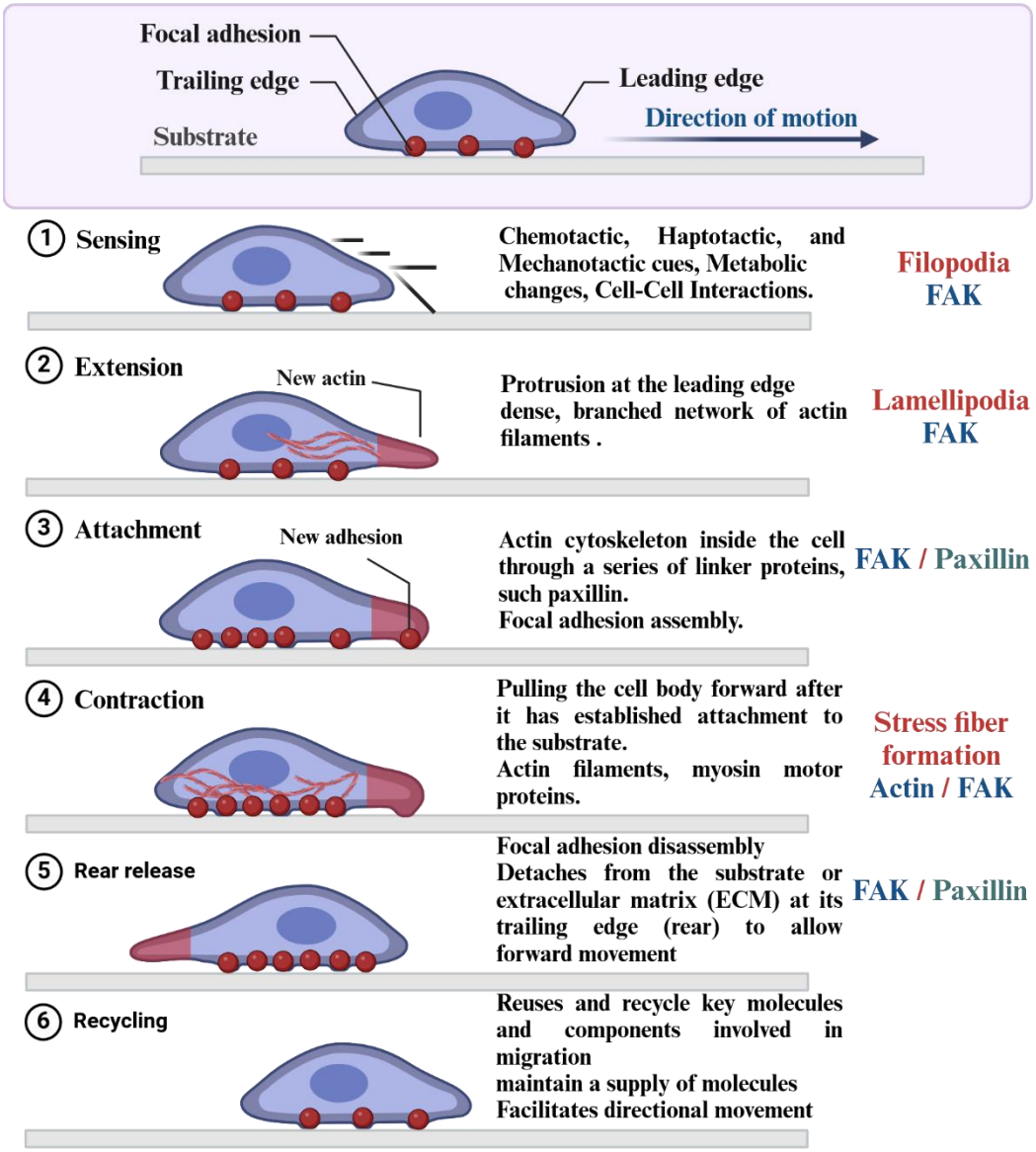


Figure 1.11: Major steps of cell migration. (1) the formation of filopodia via the regulation of the protein Cdc42 (2) the extension of the cell causing formation of protruding lamellipodia (3) the attachment of the protrusions to the extracellular matrix at focal adhesions (4) cell body contraction and formation of actin stress fibers allowing forward progression; (5) cell rear release by stress fiber–mediated traction forces (6) adhesive and signaling components recycling. The Illustration is created in BioRender. Bajes, F. (2024) BioRender.com/p17l086, and adopted from: Lamalice, L., Le Boeuf, F., & Huot, J. (2007). Endothelial cell migration during angiogenesis. *Circulation research*, *100*(6), 782–794. https://doi.org/10.1161/01.RES.0000259593.07661.1e`

2- Extension and cell protrusion formation:

After the cell senses the signal, extensions of the cell's leading edge is generated forcing the cell to move forward, these extensions are called lamellipodia and filopodia. Lamellipodia and filopodia are formed by the reorganization of the actin cytoskeleton inside the cells [113]. Lamellipodia can be categorized as broad, sheet-like extensions, composed of dense branched actin filaments, while filopodia are thinner projections, they extend beyond lamellipodia having unbranched actin filaments (**Figure 1.12**).[114]

The extension step is regulated by signaling proteins to ensure an accurate migration process. The two major players during the extension step are the actin and the FAK, the integration work between those two proteins controls the dynamics of the cytoskeleton and the cell adhesion to the ECM.[115] Lamellipodia and filopodia are produced when actin filaments (F-actin) after the polymerization of actin monomers (G-actin), resulting in forcing the cells to migrate forward. Some protein complexes such as Arp2/3 stimulate the actin nucleation (the initial and critical step in the **actin polymerization),** which makes it the main responsible protein for regulating the actin cytoskeleton, especially in the formation of branched actin networks[116]. The actin is continuously polymerized at the leading edge and depolymerized at the rear; this dynamic provides the appropriate driving force for the membrane to move forward during the migration[101]. Besides the major role of actin in this step, FAK integrally plays a major role too, it regulates the cell adhesion to the ECM by the focal adhesions. Focal adhesions are protein complexes that connect the actin cytoskeleton to the transmembrane receptors (integrins). When FAK is activated, it leads to the activation of other proteins (such as paxillin) involved in the regulation of actin dynamics, creating the connection between the actin cytoskeleton and integrins at the focal adhesions, creating a stable leading edge during extensions.[101]

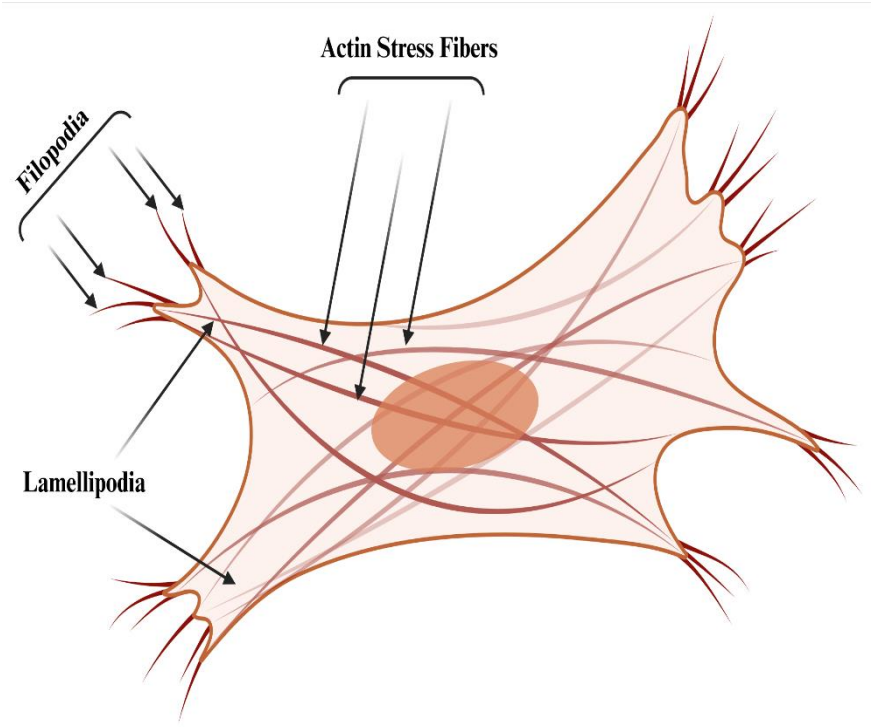


Figure 1.12: A schematic presentation of the different actin structures: Lamellipodia and Filopodia. These two protrusions are a characteristic feature at the leading edge of a migrating cells. Major role of those protrusions is pulling the cell forward during cell migration. A two-dimensional actin network forms lamellipodia. Filopodia are actin protrusions seen inside the lamellipodia. Actin-binding proteins create them by cross-linking actin filaments into bundles. Filopodia attach to the cell surface by forming focal adhesions with the substratum. Stress fibers give cells mechanical support and are created when a cell forms strong bonds with a substrate using integrins, which are transmembrane adhesion molecules. The Illustration is Created in BioRender. Bajes, F. (2024) BioRender.com/k87z851, and adopted from: Striedinger, K., VandenBerg, S. R., Baia, G. S., McDermott, M. W., Gutmann, D. H., & Lal, A. (2008). The neurofibromatosis 2 tumor suppressor gene product, merlin, regulates human meningioma cell growth by signaling through YAP. *Neoplasia (New York, N.Y.), 10*(11), 1204–1212.

3- Cell Attachment: Formation of New Adhesions

After the cell extension, and to keep the directional migration well regulated, the migrating cell must establish new points of contact with ECM to build a traction force and anchor the new protrusions (lamellipodia and filopodia), the focal adhesions will mediate this attachment, doing the role of both signaling hubs and physical anchors. The

integrins will work as the linkers between the ECM and the intracellular actin cytoskeleton to facilitate the cell attachment, via activating the FAK and paxillin to

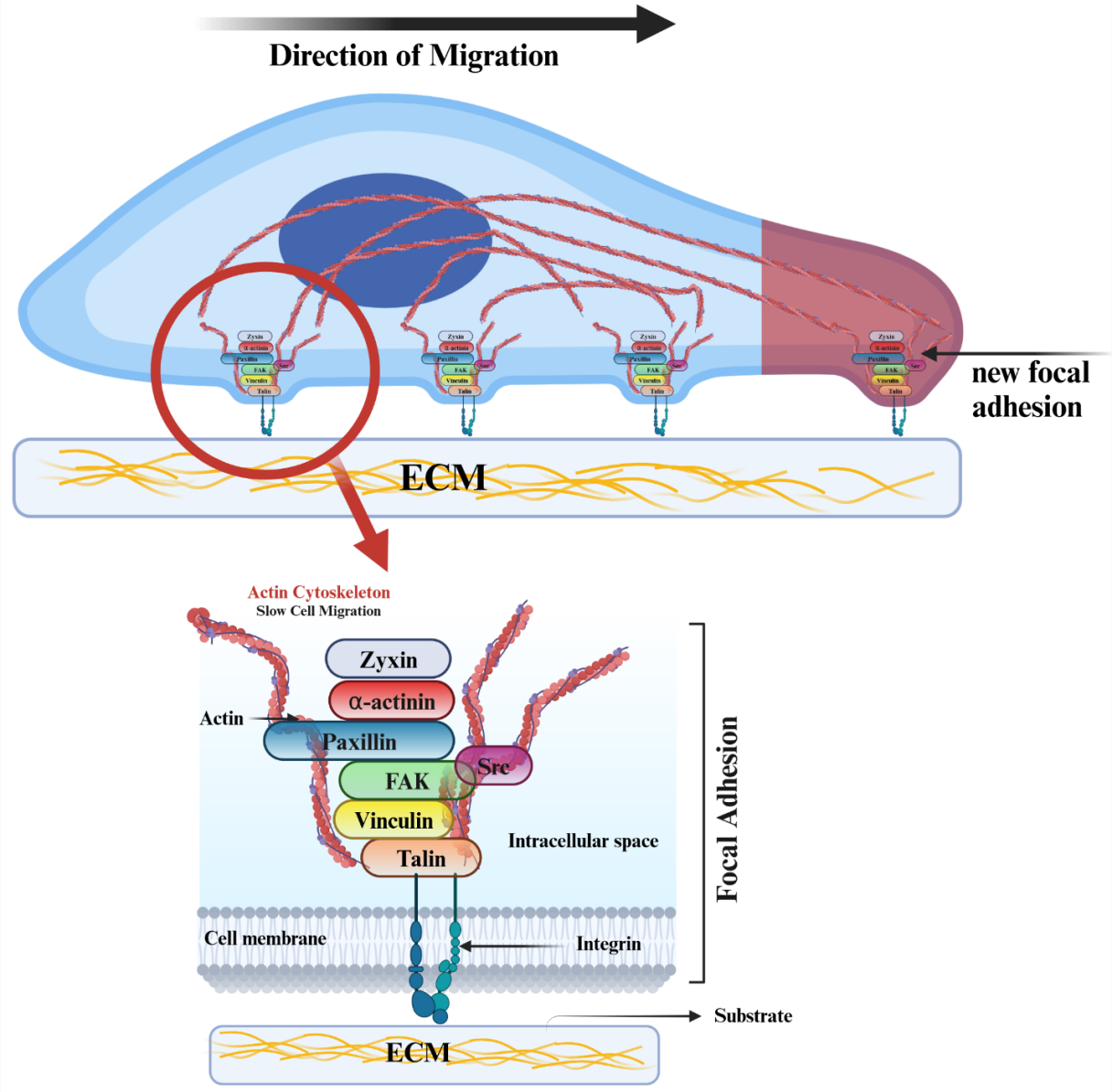


Figure 1.13: A schematic representation of cell attachment during migration. The direction of migration and sequence of adhesion-deadhesion steps in normal regulated situations should be adhesion (attachment) in the leading edge, de-adhesion (detachment) in the rear of the cell. attachment on the leading end of the cell begins with integrin subunits binding to domains in the ECM substrate. **Followed by** clustering and the formation of a mature focal adhesion complex. **Then** Cytoskeletal interactions with the focal adhesion causing contractile forces. **Finally,** the mature focal adhesion complex at the rear of the cell disassembles, which allows the cell's trailing edge to detach, effectively retracting the cell body forward as it migrates. The Illustration is Created in BioRender. Bajes, F. (2024) BioRender.com/o40y511, and adopted from: Frontiers in Cell and Developmental Biology. (2019). *Cell adhesion and migration*. *Frontiers in Cell and Developmental Biology, 7*, Article 251, and Joo, E. E., & Yamada, K. M. (2015). Cell adhesion and movement. In A. Vishwakarma, P. Sharpe, S. Shi, & M. Ramalingam (Eds.), *Stem cell biology and tissue engineering in dental sciences* (pp. 61–72). Academic Press.

stabilize the attachment of the cell (**Figure 1.13**).[102,117]

4- Contraction: Cell Body Movement

Following the leading-edge attachment, the cell's body moves forward through contraction caused by "actomyosin contraction" (**Figure 1.14**). Actomyosin is the interaction between actin filaments and myosin II resulting in the production of the mechanical force needed to contract the cell body.[118] The F-actin (a polymerized form of G-actin) is a scaffold for myosin II protein, the interaction between those two proteins are vital for the contractile activity that caused in turn to activate a protein called Rho-associated protein kinase (ROCK) downstream of Rho GTPase, which eventually end up in causing the myosin II Phosphorylated, leading to promoting contraction of actin fibers, generating forces that enable cells to be displaced.[119,120]

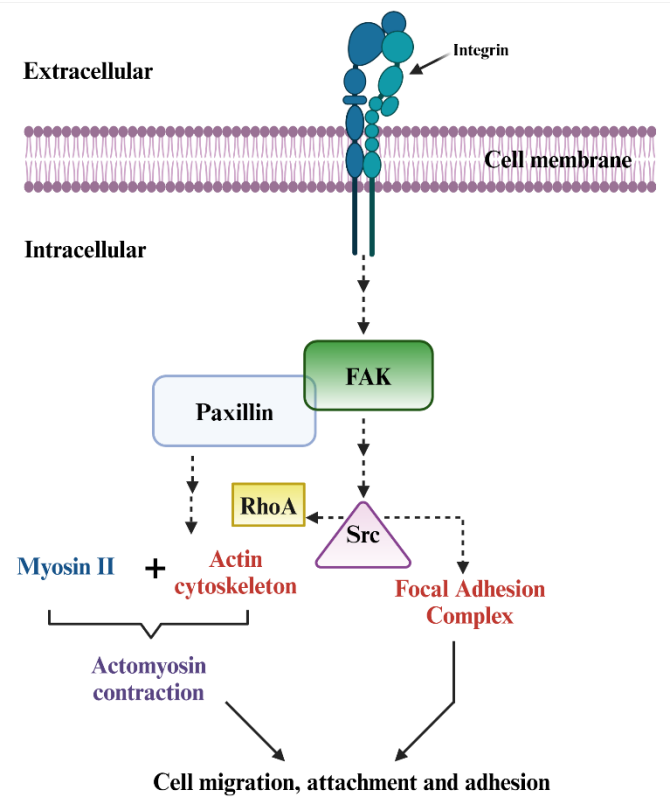


Figure 1.14: Illustration for simplified signaling pathways of FAK and PPAXillin controlling the actin cytoskeleton rearrangement, which is connected to the cell attachment, detachment, contractile and migration as a collective process. The Illustration is created in BioRender. Bajes, F. (2024) BioRender.com/g82b880, and adopted from: Wei, H., Malik, M., Sheikh, A. M., Merz, G., Ted Brown, W., & Li, X. (2011). Abnormal cell properties and down-regulated FAK-Src complex signaling in B lymphoblasts of autistic subjects. *The American journal of pathology*, *179*(1), 66–74. https://doi.org/10.1016/j.ajpath.2011.03.034

5- Rear Release: Detachment From the Back:

The focal adhesions (FAK and paxillin) act as anchoring points in the rear (tailing edge), the cell must release its adhesions at the rear (trailing edge) from the ECM to proceed to forward movement. FAK is the key regulator of the focal adhesion turnover. When the lamellipodia and filopodia pull the cell forward, FAK is activated (autophosphorylation at Tyr 397) at the trailing edge, this step is followed by additional phosphorylation on additional sites on the FAK via the interaction with other proteins such as Src. At the same time, the activation of FAK triggers the disassembly of focal adhesions at the rear sites by initiating downstream signaling pathways that regulate cytoskeletal dynamics and proteolytic activity. This disassembly occurs via the phosphorylation of paxillin and other substrates, besides the recruitment of proteins that mediate adhesion disassembly and cytoskeletal remodeling. This action allows the cell to detach from the ECM at the rear, making retraction possible.[121,122,123,124] Several proteins, including Akt1, calpain2, and microtubules, directly contribute to the breakdown or disassembly of focal adhesions. By phosphorylating FAK at particular locations, Akt1 stimulates the disassembly of focal adhesions and increases cell motility. Calpain2 activity, on the other hand, starts the proteolytic cleavage of focal adhesion components, which leads to their disassembly. On the other hand, microtubules often target and disrupt focal adhesion growth, particularly toward the cell's rear, which promotes their disassembly. These findings suggest that targeting these proteins could effectively regulate focal adhesion dynamics throughout tumor cells. [125,126,127]

6- Recycling: Reuse of Cellular Components:

In a cellular context, recycling means the reuse of cellular components, and its a critical process for maintaining cellular homeostasis, resource efficiency, and proper function. Cells use different mechanisms to recycle molecules, organelles, and macromolecules. This process allows cells to recover resources during stress conditions. Recycling during cell migration is the final step which involves recycling membrane components, integrins, and other adhesion proteins from the rear to the leading edge, enabling continuous migration. [128,129]

1.9 Modes of Cellular Movement: Collective Migration, Directional Migration, and Random Motility

According to the literature, cells move by one of three primary migration styles: collective migration, directional migration, and random motility. Each plays a unique role in biological processes like angiogenesis and cancer progression. [130,131]

Collective migration is defined as the movement of groups of cells together in a coordinated fashion, maintaining cell-cell junctions and communication. Collective cell migration is involved in many biological processes such as angiogenesis, allowing structured vascular network formation by migrating as a cohesive unit. This type of migration is crucial for the integrity and stability of new blood vessels during their formation. In cancer, collective migration enables more efficient invasion into surrounding tissues and protection from immune responses. In this mode of migration, cells must establish a pyramid of cellular identities, or in other words: choose a “leader” and “follower” cells for this process to be well regulated. [132,133]

Directional migration refers to the well-guided and regulated of individual cells towards a specific signal (such as growth factor) and direction (wound gap).[134] Endothelial cells respond to angiogenic factors such as VEGF via directional migration, guided to the sites where new blood vessels are needed[135]. When it comes to cancer progression, directional migration allows cancer cells to invade tissues and thus effective metastasis occurs[136]. For cells to migrate towards a signal directionally, they should be able to recognize cues in the surrounding environment, and migrate towards or away from it, causing cells to repair damaged tissues and navigate an immune response. Failure of cells to migrate in a directional way may lead to defects and disorders during normal tissue development and immune deficiencies. [111]

The uncoordinated (with no direction) movement of cells is called random motility.[131] Random migration occurs when the cell has relatively low intrinsic directionality, this means if the cell lacks the organized internal mechanisms that drive them to a specific direction, their movement is less structured and more exploratory or random. [137] Random motility allows immune cells to explore tissues and detect abnormal cells or injuries,[138] helping cancer cells initiate the early stages of invasion before transitioning to more directed migration modes.[139]

1.10 The Significance of This Project

This project introduces qualitative and quantitative data as part of the efforts in the research field to get steps closer to the development of novel anticancer treatments by unraveling how lactate and protons influence critical processes like cell migration, adhesion, and attachment in both human umbilical vein endothelial cells (HUVEC) and murine melanoma cells (B16F10).

Why lactate and protons? Tumor cells generate high levels of both species as a result of their modified metabolism, creating an acidic microenvironment that promotes aggressive cancer behavior, including enhanced metastasis. By investigating these metabolic byproducts affecting the cancer cells and endothelial cells (essential for angiogenesis that cancer cells depend on for growth and spread), this project provides accurate data about the effects of both species on the underlying mechanisms driving tumor progression. The data produced in this project add another piece of the puzzle of understanding the tumor microenvironment, which will help _ besides other research in the field_ in designing targeted therapies that disrupt tumor cell migration and attachment to surrounding tissues, offering new strategies to disrupt the metastasis and improve cancer treatment outcomes.

Chapter 2: Materials and Methods

2.1 Reagents and Chemicals

The fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), Endothelial growth medium-2 (EGM-2), Dulbecco's phosphate buffered solution (DPBS), and trypsin/EDTA were from Lonza (Walkersville, MD). Penicillin-Streptomycin (100X), Rhodamine-phalloidin, Dimethyl sulfoxide (DMSO), NuPAGE™ 4 to 12%, Bis-Tris, 1.0–1.5 mm, Mini Protein Gels, Super Signal™ West Atto Ultimate Sensitivity Substrate and the Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP from Thermo Fisher. were from Thermo Fisher. NuPAGE™ MES SDS Running Buffer (20x), Invitrogen™ NuPAGE™ Transfer Buffer (20X), and Sodium L-lactate, 98+% were from Invitrogen by Thermo Fisher Scientific. 4-(2-Hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES), and 2-(4-morpholino)-ethane sulfonic acid (MES) were from Sigma life science (St. Louis, MO). N-(2-hydroxyethyl)- piperazine-N0-3-propanesulfonic acid (EPPS) and Pierce ™ RIPA Buffer were from Thermo Scientific. Total paxillin (cat#: 50195S), total focal adhesion kinase (cat#: 3285), Phospho-FAK (Tyr397) Rabbit mAb (cat#: 3283), pPAX (Y118) (Tyr118) Rabbit mAb (cat#: 69363), and GAPDH Rabbit mAb (cat#: 5174) were from Cell Signaling, Inc. Nitrocellulose Membranes (0.2μm) were from Bio-Rad. Blotting Papers were from VWR®.

2.2 Cell Lines

The B16F10 cell line used for the investigation in this study was maintained in DMEM supplemented with 10% FBS (nutrient factor) and 1% Penicillin-Streptomycin-100X (antibiotics). The HUVEC cell also used in this study was maintained in EGM-2. EGM-2 is basal medium mixed with 2% FBS (nutrient factor), 0.04% Hydrocortisone (anti-inflammatory factor), 0.4% hFGF-B (Mitogenic Activity factor), 0.1% VEGF (Endothelial Cell Survival and Proliferation factor), 0.1% R3-IGF-1 (Cell growth), 0.1% Ascorbic Acid (Antioxidant), 0.1% hEGF (cell proliferation factor), 0.1% GA-1000 (Antibiotic and Antifungal), and 0.1% Heparin (Anti-coagulant and Binding Sites for Growth Factors). Normal cell culture cells were 95% alive, had a passage number less than 8, and could only be grown to 85-90% confluence (**Figure 2.1**). For assays used in this project, all cells were cultured in a humidified tissue culture incubator at 37 ^{0}C with 5% CO_2. 7.5 mM HEPES, 7.5 mM EPPS, and 7.5 mM MES (together referred to as HEM) were used in conjunction with NaOH and HCl to buffer the media to the required pH for investigations involving pH fluctuation.

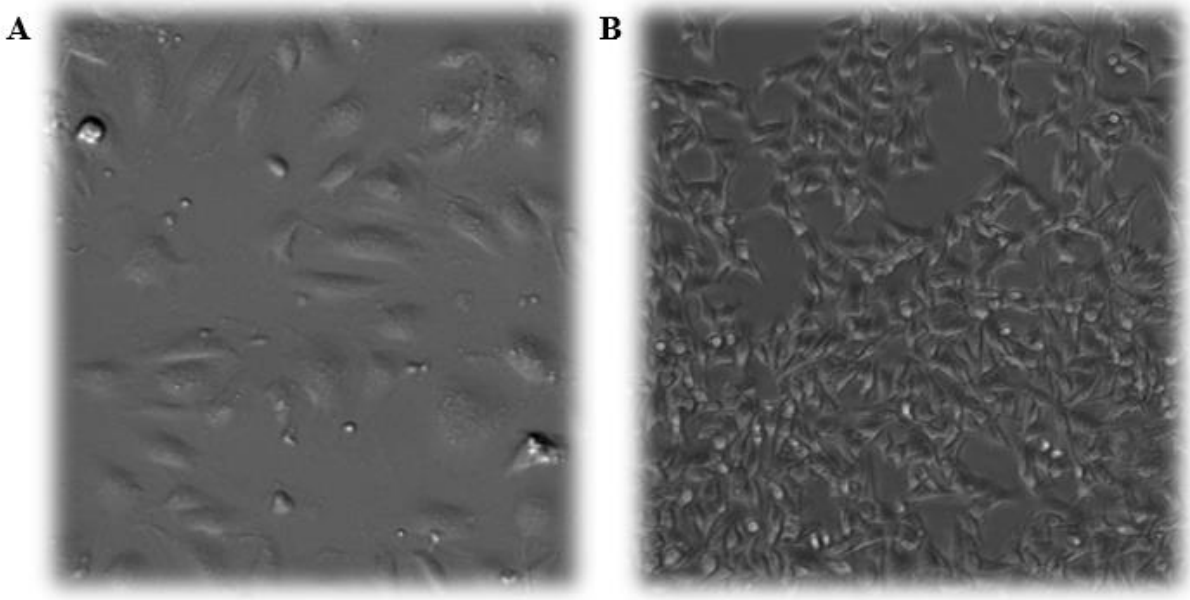


Figure 2.1: (A) HUVECs image taken by ZEISS Cell discoverer 7 microscope with a 10X objective and (B) B16F10 cells image taken by ZEISS Cell discoverer 7® microscope with a 5X objective at 90% confluency.

2.3 Wound Healing Assay and Cell Migration

HUVEC ($1*10^4$ cell/well) and B16F10 ($2.5*10^4$ cell/well) were seeded in 48-well plates and allowed overnight to attach and grow to confluency. Cells the next day should be in a uniformed monolayer confluency. A 100 µL pipette tip was then used to create a wound through the center of the confluent cell layer. Cells were next treated with 500 µL of the appropriate pH buffered growth treatment media, DMEM + 10% FBS + (0mM, 1mM, 10mM or 40mM L-Lactate) for B16F10 cells, or EGM-2 + (0mM, 1mM, 10mM or 40mM L-Lactate) media for HUVECs. Videos were taken using ZEISS Cell Discoverer 7 (CD7). Photos at specific time points were extracted and analyzed using Zen 3.4 (blue edition) software. To quantify the data, the wound size at the start of the assay and comparison to the wound size at the end of the assay was done in addition to the rate of gap closure (or the rate of collective migration), which is a measure of the speed of the motion of the cells or the (gap closure rate) that can be measured under different conditions. The rate for each treatment was represented by a bar graph and tested for statistical significance using one-way ANOVA followed by Tukey's multiple comparisons using GraphPad Prism 5 software.

2.4 Tissue Culture Plate Attachment (Single Cell Attachment) Assay

Both HUVECs and B16F10 cells were detached using 0.25% Trypsin-EDTA. 2×10^4 cells were resuspended in the assigned microenvironment in 24-well plates. The buffers were prepared using: DMEM / (HEPES, MES, EPPS) with 10% FBS, and (0. 1. 10. 40 mM) Lactate buffered pH 6.4 and 7.4 for transformed cells, and EGM-2 / (HEPES, MES, EPPS) with 5% FBS, and (0. 1. 10. 40 mM) Lactate buffered pH 6.4 and 7.4 for HUVEC. Plates were incubated

at 37^0C and 5% CO_2 for an hour. This time was enough to ensure the attachment of the majority of the cells, this was checked by shaking the plate manually by hand under the microscope and observing the movement of cells. An average of 10 images taken with an objective of 10X were quickly taken using the EVOS® fl digital inverted microscope in several areas for the wells. The images were used to quantify the results of cell spreading under different conditions. The results of cell spreading, and attachment were quantified by classifying the cells in the images as round, spread, and polarized (**Figure 2.2**). Round cell was characterized by the cell shape with no cell protrusion (more like a circle shape), while the spreading cell was characterized by having at least one membrane protrusion, and the polarized cell was characterized by having a polarized cell body, having lamellipodium and a tail. The different morphological shapes for each cell line were quantified by dividing the number of round, spread, and polarized cells by the total number of cells counted in the images taken. The average percentages were represented by a bar graph and tested for statistical significance using one-way ANOVA followed by Newman-Keuls multiple comparison test using GraphPad Prism 5 software.

2.5 Single-Cell Migration (Cell Morphology) Assay

HUVECs and B16F10 cells were detached using 0.25% Trypsin-EDTA. 2 x 10^4 cells were resuspended in the assigned microenvironment in 24- well plates. The buffers were prepared using: DMEM/ (HEPES, MES, EPPES) with 10% FBS, and (0. 1. 10. 40 mM) lactate buffered pH 6.4

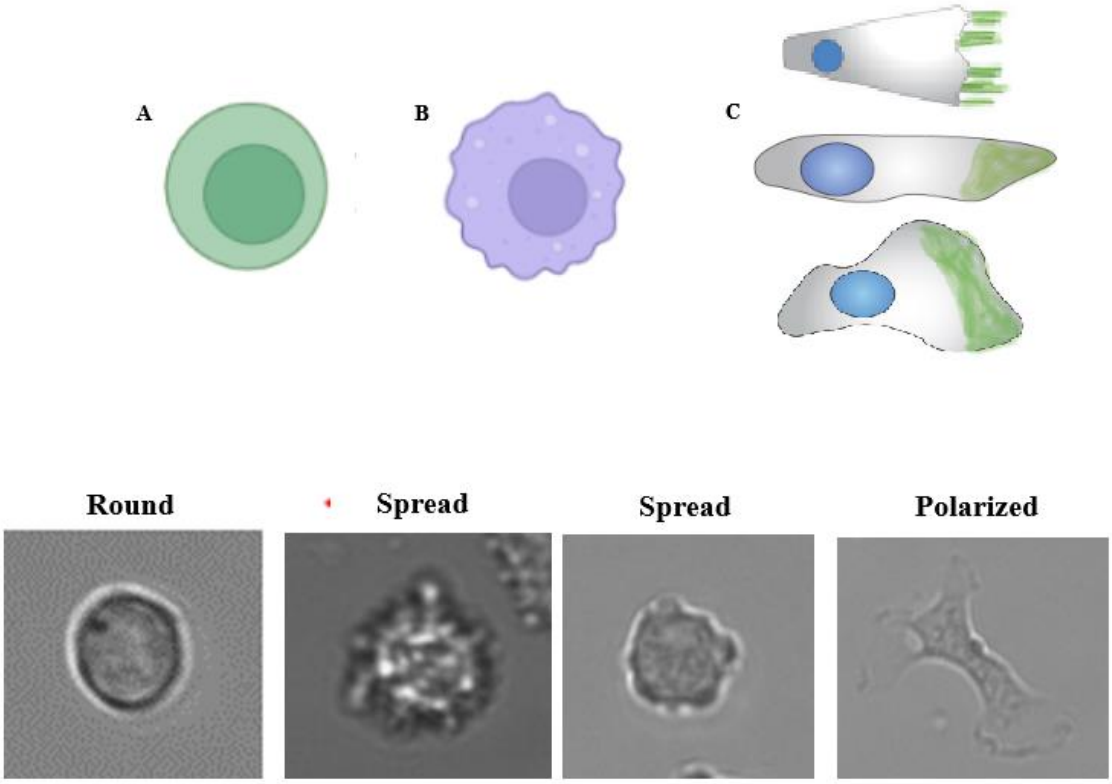


Figure 2.2. Illustration for morphologies of the three classified cells' morphology for the attachment assay quantification. (A) round-shaped cell (B) spread-shaped cell (C) polarized-shaped cell.

and 7.4 for transformed cells, and EGM-2 / (HEPES, MES, EPPES) with 5% FBS, and (0. 1. 10. 40 mM) lactate buffered pH 6.4 and 7.4 for HUVECs. Plates were incubated at 37^0C and 5% CO_2 overnight to ensure attachment and normal migration. Then treatments were added, and cells were incubated overnight. 10-15 images were taken using the ECHO revolve microscope in several areas for the wells. The images were used to quantify the results of elongation represented by the polarizing Index (**Figure 2.3**) under the different treatment conditions. The polarizing index is the ratio of the cell length including the longest protrusion divided by the cell width. The cell lengths and polarizing index were represented by a scatter plot graph and tested for statistical significance

using one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism 5 software.

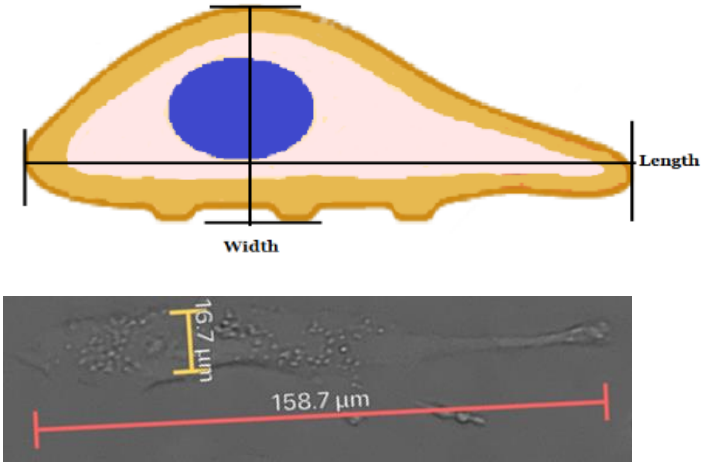


Figure 2.3. polarizing index is the ratio of the cell length including the longest protrusion divided by the cell width.

2.6 Cell Motility Tracking in Wound Scratch Assay using FIJI®

(Directional Migration and Random Motility)

Both HUVEC ($1*10^4$ cell/well) and B16F10 ($2.5*10^4$ cell/well) cells were seeded in 48-well plates and allowed overnight to attach and grow to confluency. Plates were incubated at 37^0C and 5% CO_2 overnight to ensure attachment and normal migration. Cells the next day should be in a uniformed monolayer confluency. The buffers were prepared using: DMEM/ (HEPES, MES, EPPS) with 10% FBS, and (0, 1, 40 mM) lactate buffered pH 6.4 and 7.4 for B16F10 cells, and EGM-2 / (HEPES, MES, EPPS) with 5% FBS, and (0, 1, 40 mM) lactate buffered pH 6.4 and 7.4

for HUVECs. A 100 µL pipette tip was then used to create a wound through the center of the confluent cell layer. Then treatments were added, and half the amount of buffer in each well was changed every 2 hours assuring the pH stability. Timelapse videos were taken using the Zeiss Cell Discovery 7. The videos were processed for tracking assay using Fiji® Image J, and the migration path of representative cells at the edge (leading or tip cells) of the wound in each well was tracked manually. The directional migration and random motility (**Figure 2.4**) data were represented by a scatter plot and bar graph. Results were tested for statistical significance using one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism 5 software.

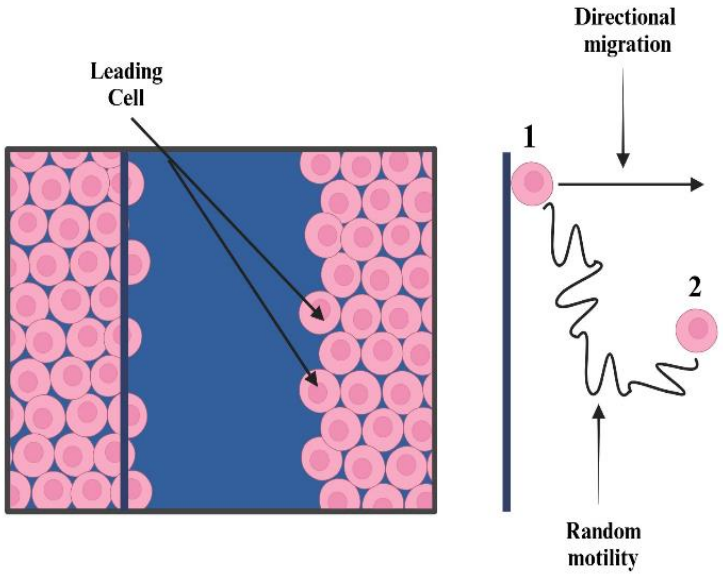


Figure 2.4. The illustration represents directional migration and random motility.

2.7 Phospho Paxillin (Y118), Phospho-Focal Adhesion Kinase (Y397) Immunocytochemistry (ICC) and Actin staining.

HUVECs and B16F10 cells were detached using 0.25% Trypsin-EDTA. Cells were resuspended in the assigned microenvironment in 48-well plates (based on the assay: wound scratch assay, single cell attachment assay, or cell morphology assay). Plates were incubated at 37^0C and 5% CO_2 for the required time based on the assay. Next, media was aspirated, and wells were washed using DPBS (Lonza®). Cells were fixed for 10-13 min using 4% paraformaldehyde solution, followed by washing the cells twice with DPBS, followed by 0.1% Triton-X permeabilization buffer (0.1% Triton-X in DPBS) added to each well and incubated at room temperature for 15 minutes then washed with DPBS. The cells then were blocked by 1:20 dilution of goat serum in PBS-T (0.1% Tween-20 in 10X DPBS) blocking solution at room temperature for 1 hour. The blocking solution was then aspirated followed by adding the primary antibody of interest {pPAX (Y118), or phospho-focal adhesion kinase (Y397) from Cell Signaling Technology® diluted in the blocking serum 1:50} into each well. It was then placed on the shaker in the 4° C cold room overnight. The next day, the primary antibody was removed from the wells, followed by three times washing with DPBS, followed by adding goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 430, 1:200 dilution for a 1-hour incubation at room temperature. The secondary was then aspirated, and the wells were then three times washed with DPBS. Subsequently, each well was stained with 100nM Rhodamine Phalloidin in DPBS for actin staining, and the tissue plate was covered with aluminum foil for 1 hour at room temperature. Then the media was aspirated, and wells were washed three times with DPBS (each time 5 min), then one drop of

VECTASHIELD® Mounting Medium for fluorescence with DAPI was added to each well. Lastly, imaging was done using the ECHO revolve microscope in several areas for the wells.

2.8 Western Blot

Cells were cultured in $10cm^2$ (from VWR tissue culture dishes, cat#: 10861-680) with 85% confluency, then washed with cold phosphate-buffered saline (PBS), then aspirated and carefully soaked up any extra DPBS. Next, total proteins were extracted using a 250µl RIPA lysis buffer (with Phosphatase and protease inhibitors). Samples were incubated in the ice for 20-25 min over the shaker. Cell scrapers to collect the lysate cells in the culture plate, then lysates were transferred to 1.5 ml labeled microcentrifuge tubes, centrifuged at 14,000 g for 15 min at 4 C^0, then transferred the supernatant to a new tube, Aliquot and stored at -20C^0. Protein concentration was quantified by the DC protein assay (BIO-RAD ®). The same amount of proteins was separated through SDS-PAGE, and the gels were transferred onto nitrocellulose membranes for Western blotting. Nitrocellulose membranes were washed using 1X Tris-Buffered Saline 0.1% Tween® 20 Detergent (TBST), then blocked using 5% Bovine Serum Albumin (BSA) dissolved in (TBST) for 1 h at room temperature. Next membranes were incubated with primary antibody overnight in 4C^0 with a gentle shake. The next day, membranes were washed 3X for 15 min each wash using TBST. Antigen–antibody complexes were detected with IgG (H+L) Cross-Adsorbed Secondary Antibody HRP and were revealed using an ECL kit (Super Signal™ West Atto Ultimate Sensitivity Substrate). Quantification of the bands was done by Image J (Fiji) software. Data was represented by a scatter plot and bar graph. Results were tested for statistical significance using one-way ANOVA followed by Newman-Keuls multiple comparison test using GraphPad Prism 5 software.

2.9 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software, Inc., Irvine, CA) and represented as mean ± SEM. Statistical significance was measured using one-way ANOVA followed by Tukey's or Newman-Keuls multiple comparison test. Error bars represent a standard error in all graphs. *** $P < .001$, ** $P < .01$, * $P < .05$, ns $P > .05$.

Chapter 3: Results

3.1 Acidity reduced collective cell migration for HUVEC and B16F10 cells, and the collective migration is further reduced by the combined effect of acidity and lactate.

A wound-healing assay was performed to assess the cellular migration of HUVECs and B16F10 under the effect of an acidic microenvironment with and without lactate. Time-lapse videos were taken using Cellular Discovery microscope CD7® by Zeiss. The experiment lasted 13 hours for HUVECs and 11 hours for B16F10 cells until the wound was nearly closed. The collective migration rate was calculated by dividing the distance traveled during the time course of the experiment over the time of the experiment. General qualitative data analysis clearly showed a delayed collective migration rate in both cell models (**Figure 3.1A, 3.1B**), with the difference of greater significant delay in HUVECs, when cells were under the effect of acidosis compared to physiological pH treatment. Moreover, the quantitative data analysis for the captured videos showed that the reduction in collective migration was further significant when cells were under the combined effect of acidosis and lactate (**Figure 3.1C, 3.1D**). For HUVECs, the reduction in collective migration was 31.7% when cells migrated under acidosis compared to cells treated with physiological buffered media. The reduction was further signified when cells treated with acidic media with 40mM lactate showing a 74.5% reduction in collective migration compared to cells migrating in normal pH microenvironment. For B16F10 cells, the reduction was 29% when cells migrated under acidosis compared to cells treated with pH 7.4 buffered media. The reduction was further signified when cells treated with acidic media with 40mM lactate showing a 53% reduction in collective migration compared to cells migrating in normal pH microenvironment.

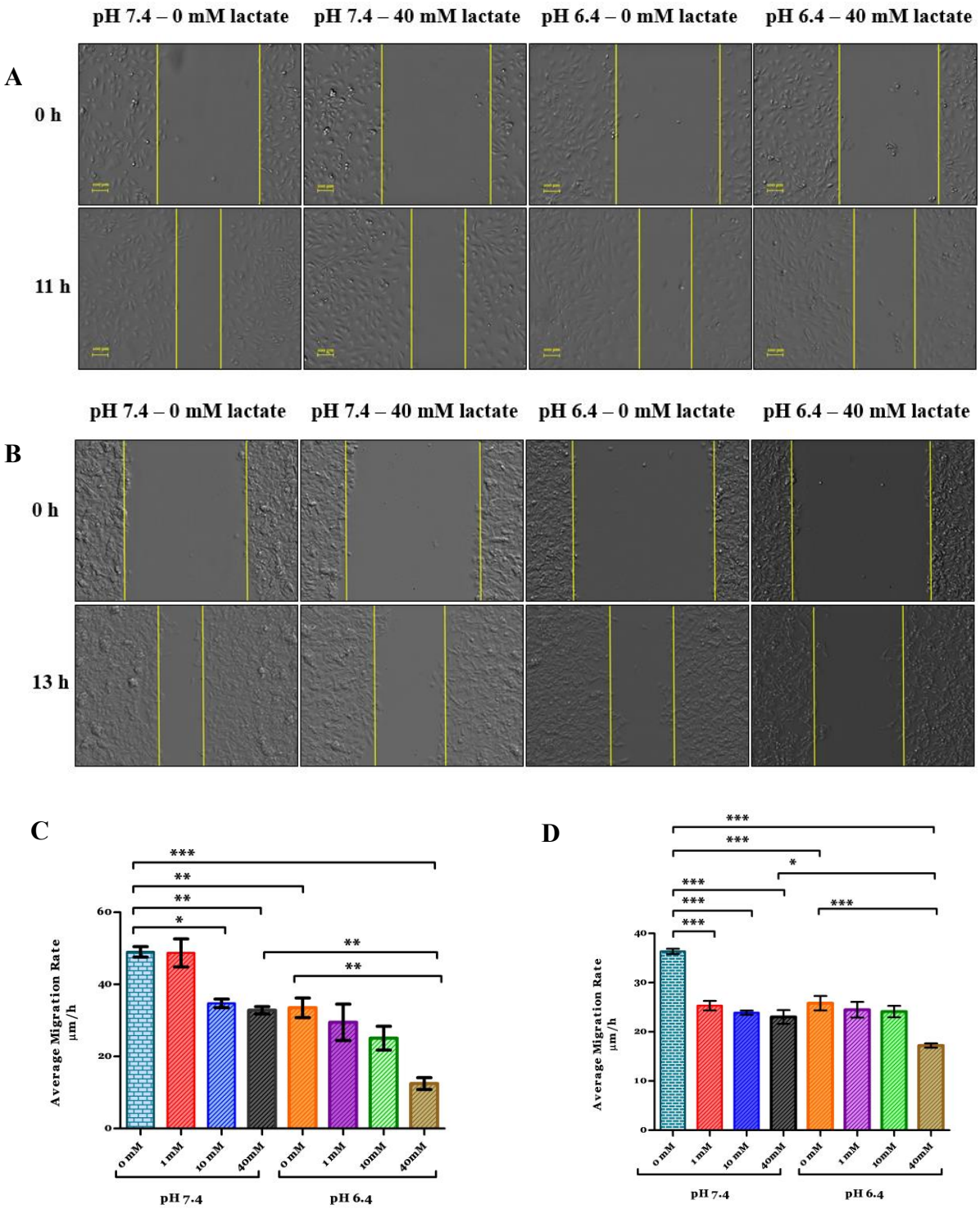


Figure 3.1. Qualitative and quantitative data from in vitro scratch wound healing assays for **HUVECs** and **B16F10** Cells **(A)** and **(B)** Representative images for HUVECs and B16F10 respectively. Cells migrating into the cell-free region are decelerated when incubated in 6.4, compared to 7.4 medium. Images show a further reduction effect for the high lactate level accompanied by acidity. Images were extracted from a time-lapse video using the CD7 microscope from Zeiss. **(C)** and **(D)** quantitative time-course analysis of a wound healing assay (13 hours for HUVEC, 11 hours for B16F10). Each experiment was performed five times with five separate biological replicates (n=5). Scale bar for all images is 100µm. The rate for each treatment was represented by a bar graph and tested for statistical significance using one-way ANOVA followed by Tukey's multiple comparisons using GraphPad Prism 5 software (*** $P < .001$, * $P < .05$, ns $P > .05$).

3.2 Acidity reduced directional cell migration for HUVEC and B16F10 cells, and the directional migration is further reduced by the combined effect of acidity and lactate.

A wound-healing assay was conducted to evaluate the directional migration of HUVECs and B16F10 cells located at the wound edges, known as tip or leading cells, in an acidic microenvironment with and without lactate. Time-lapse videos were taken using Cellular Discovery microscope CD7® by Zeiss. The experiment lasted 13 hours for HUVECs and 11 hours for B16F10 cells, until the wound was nearly closed. The directional migration for the cells was calculated by measuring the horizontal distance traveled towards the wound gap by the tip cell, using Zen 3.4 blue edition software (**Figure. 2.4**). Qualitative data analysis (**Figure 3.2A**) clearly showed a reduction in directional migration for both HUVECs and B16F10 cells when treated with acidic buffered media compared to treatment at physiological pH. The directional migration was further reduced when cells were treated with both acidity and lactate-buffered media. Quantitative analysis for HUVECs (**Figure 3.2B**) showed a 19% decrease in directional migration under acidic conditions compared to physiological pH, and a 32.6% reduction when acidosis was combined with 40 mM lactate. For B16F10 cells (**Figure 3.2D**), directional migration decreased by 29% under acidic conditions and by 44.3% when exposed to both acidic media and 40 mM lactate. The scatter plots (**Figure 3.2C, 3.2E**) were generated to assess the distribution and spread of the values, in order to understand the consistency and the variability of the response under different microenvironment treatments. For both HUVECs and B16F10 cells, the scatter plot data distributions showed different patterns in cell migration across various pH and lactate conditions. At pH 7.4 with 0 mM lactate, cells show the highest and most variable migration distances, suggesting that a physiological pH without lactate allows for optimal and diverse migratory

responses. However, when lactate added at the same pH (7.4, 40 mM lactate), migration distances decrease, and the data points are more tightly grouped, indicating a more uniform inhibitory effect of lactate on cell directional migration under non-acidic conditions. A shift from pH 7.4 with 0 mM lactate to pH 6.4 with 0 mM lactate results in a substantial decrease in migration distance, with a narrower data distribution. The effect of acidity becomes even more noticeable when combined with lactate (pH 6.4, 40 mM lactate), where the data points cluster tightly at the lowest range of migration distances, indicating that both factors together caused a highly migration-restrictive effect for cell migration.

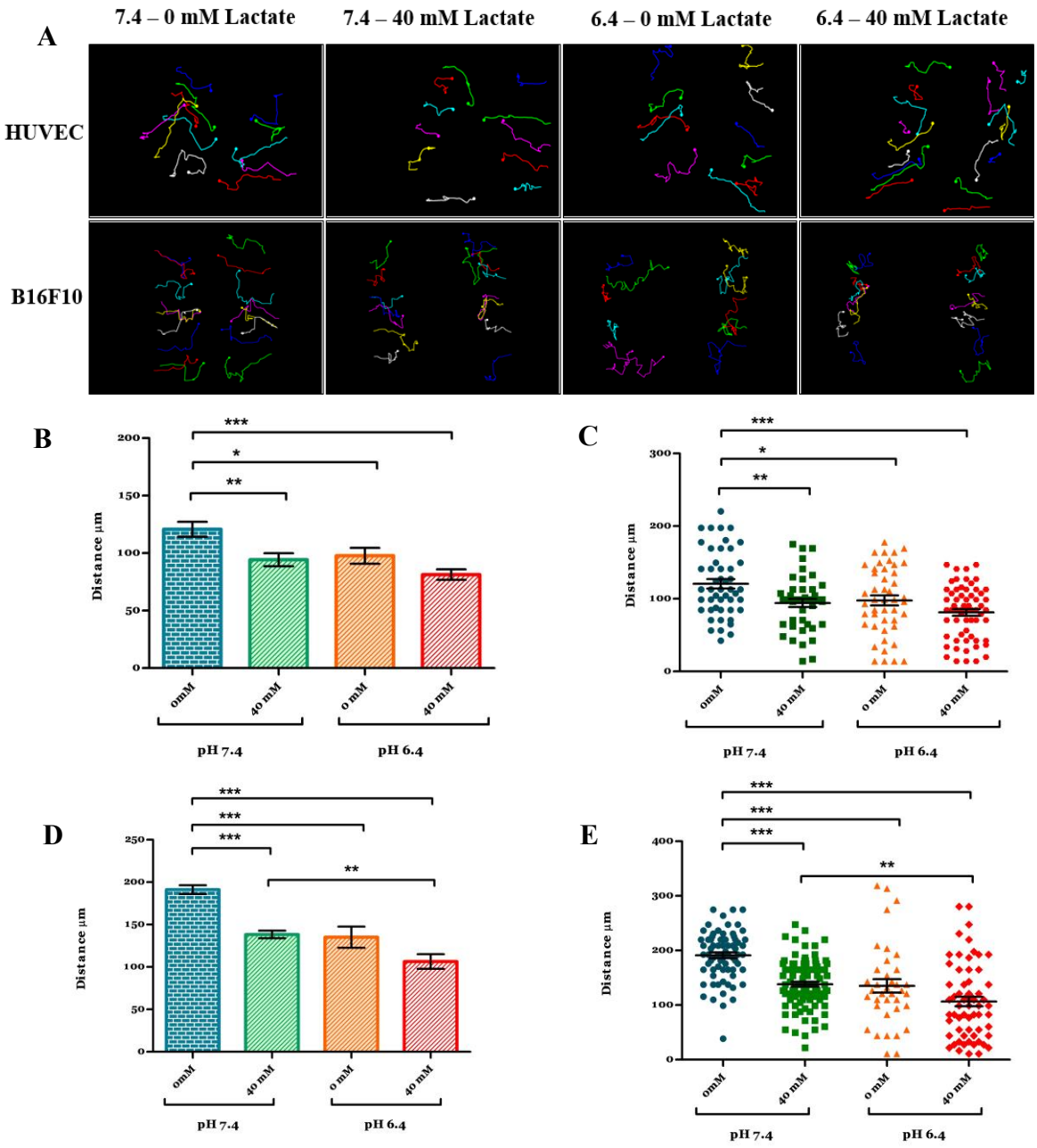


Figure 3.2. Qualitative and quantitative data for **HUVECs** and **B16F10** Cells' directional migration **(A)** Qualitative images showing the reduction in directional migration for both cell models when treated with acidic buffered media compared to treatment at physiological pH, and the further reduction when cells were treated with both acidity and lactate-buffered media. Images were taken using the CD7 and analyzed using Zen. 3.4 Blue Edition ® software. **(B)** and **(C)** quantitative time-course analysis of the directional migration for HUVEC over 13h time course, represented in a bar graph and scatter plot respectively. **(D)** and **(E)** quantitative time-course analysis of the directional migration for B16F10 over 11h time course, represented in a bar graph and scatter plot respectively. Each experiment was performed five times with five separate biological replicates (n=5) with a total of 230 HUVECs and 335 B16F10 cells. The directional migration rate for cells was represented by a bar graph and scatter plots respectively. Data was tested for statistical significance using one-way ANOVA followed by Tukey's multiple comparisons using GraphPad Prism 5 software (*** $P < .001$, * $P < .05$, ns $P > .05$).

3.3 Acidity decreases the random cell motility (total traveled path) for HUVEC, but the opposite was observed for B16F10 cells. Lactate further reduces the random cell motility when combined with acidity.

A wound-healing assay was conducted to evaluate the directional migration of HUVECs and B16F10 cells located at the wound edges, known as tip or leading cells, in an acidic microenvironment with and without lactate. Time-lapse videos were taken using Cellular Discovery microscope CD7® by Zeiss. The experiment lasted 13 hours for HUVECs and 11 hours for B16F10 cells, until the wound was nearly closed. The random cell motility for the cells was calculated by measuring the whole path traveled towards the wound gap by the tip cell, using Zen 3.4 blue edition software (**Figure. 2.4**). Qualitative data analysis (**Figure 3.3A**) showed a reduction in random motility for HUVECs when treated with acidic buffered media compared to treatment at physiological pH, and interestingly, the opposite was observed for B16F10. A further reduction was observed in random motility for HUVECs, and a reverse effect was observed in B16F10 when lactate present in acidic microenvironment. Quantitative data analysis for HUVECs (**Figure 3.3B**), random motility decreased by 10.5 % under acidic conditions compared to physiological pH, and by 30.4% when treated with acidic media containing 40 mM lactate. For B16F10 cells (**Figure 3.3D**), the increase in the random motility was 21.3% under acidic conditions compared to physiological pH and 3.8% decrease when treated with acidic media and 40 mM lactate, showing a reverse effect for lactate on transformed cells when presents in acidic microenvironment.

Both plots for HUVECs and B16F10 cells show that cells achieve the highest random motility under pH 7.4 with 0 mM lactate. the addition of 40 mM lactate at pH 7.4 results in a reduction in random motility and a tighter grouping of data points. However, the reduction is more

prominent in the HUVECs plot. Shifting to acidosis microenvironment, the randomness was reduced in HUVECs, where it became higher in B16F10 with a broader spread of data points indicating more variability in cell responses. the combination of acidic pH and lactate caused less randomness in both HUVEC and B16F10. However, even under the combined inhibitory conditions of acidosis and lactate, there is a slightly wider spread of random motility distances for B16F10, with some cells achieving moderately higher distances compared to the HUVECs random motility.

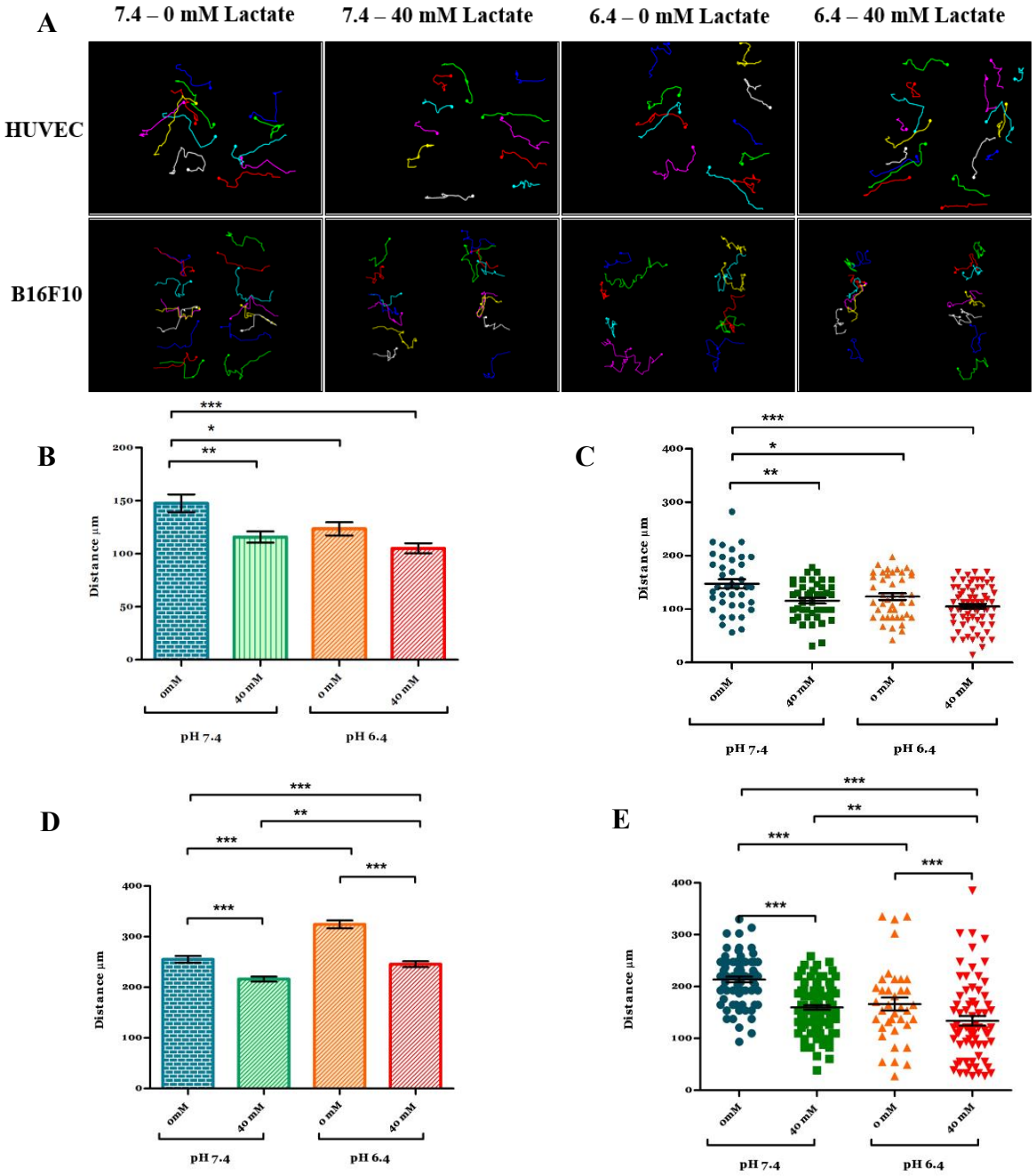


Figure 3.3. Qualitative and quantitative data for **HUVECs** and **B16F10** Cells' random motility. **(A)** Qualitative images showing the reduction in random motility for HUVECs and the increase in random motility for B16F10 when treated with acidic buffered media compared to treatment at physiological pH, and the further reduction when cells were treated with both acidity and lactate-buffered media in both cell models. Images were taken using the CD7 and analyzed using Zen. 3.4 Blue Edition ® software. **(B)** and **(C)** quantitative time-course analysis of the random motility for HUVEC over 13h time course, represented in a bar graph and scatter plot respectively. **(D)** and **(E)** quantitative time-course analysis of the random motility for B16F10 over 11h time course, represented in a bar graph and scatter plot respectively. Each experiment was performed five times with five separate biological replicates (n=5) with a total of 230 HUVECs and 335 B16F10 cells. The random motility rate for cells was represented by a bar graph and scatter plots respectively. Data was tested for statistical significance using one-way ANOVA followed by Tukey's multiple comparisons using GraphPad Prism 5 software (*** $P < .001$, * $P < .05$, ns $P > .05$).

3.4 Acidity Enhanced actin polymerization caused an increase in the formation of actin stress fibers during migration in both HUVECs and B16F10. Lactate showed an insignificant effect on the actin stress fibers formation when combined with acidity.

To further investigate the migration behavior, HUVECs and B16F10 cells were stained for F-actin. F-actin is a significant component of the cytoskeleton, it is particularly found in the lamellipodia and filopodia that promote cell movement. Staining for F-actin allows us to observe the organization and distribution of actin in the emigrating cells. Furthermore, we can directly observe the effects of acidosis and lactate in the cells' microenvironment created for this study on actin polymerization.

Cells were treated with the pH6.4 buffered media (0mM, and 40mM Lactate), and pH 7.4 buffered media (0mM, and 40mM Lactate). Rhodamine phalloidin was used to stain the F-actin in migrating HUVECs (**Figure 3.4A**) and B16F10 cells (**Figure 3.4B**). Observations showed clearly an increase in actin stress fibers and accumulation of actin in specific areas in the cell when cells were treated with an acidic buffer medium. This response can be explained by the effect of acidity on the enhancement of actin polymerization. Under physiological conditions (pH 7.4), actin stress fibers were insignificant or almost absent in either cell model. However, a significant increase in actin stress fibers was noted when exposed to high lactate concentrations combined with pH 7.4 buffered media, particularly in HUVECs. Interestingly, and more clearly in HUVECs, the combination of lactate and acidity insignificantly increases actin stress fibers compared to acidity alone, indicating that lactate didn't act relatively as a relief to the effects of acidity in this context of the assay.

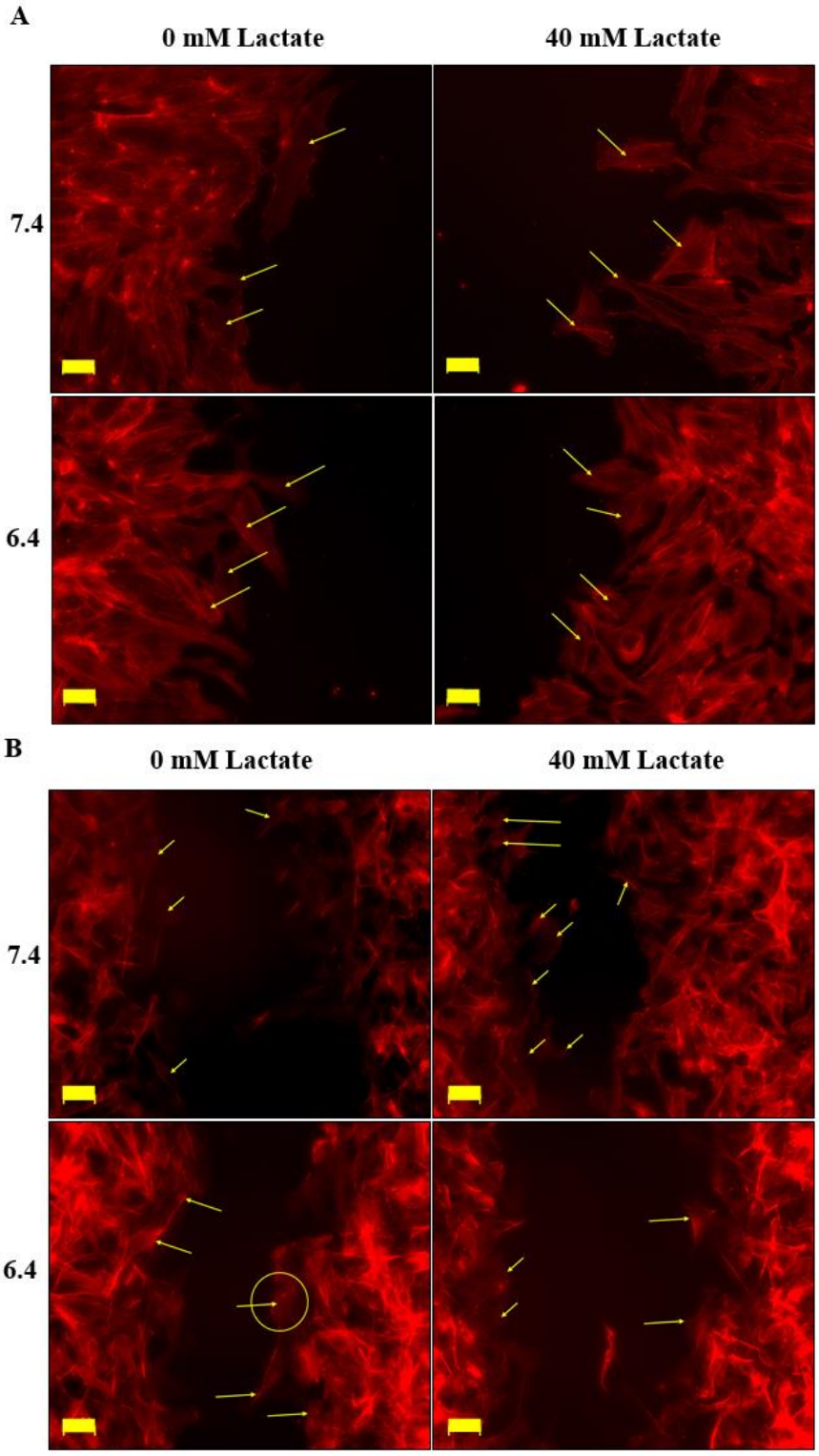
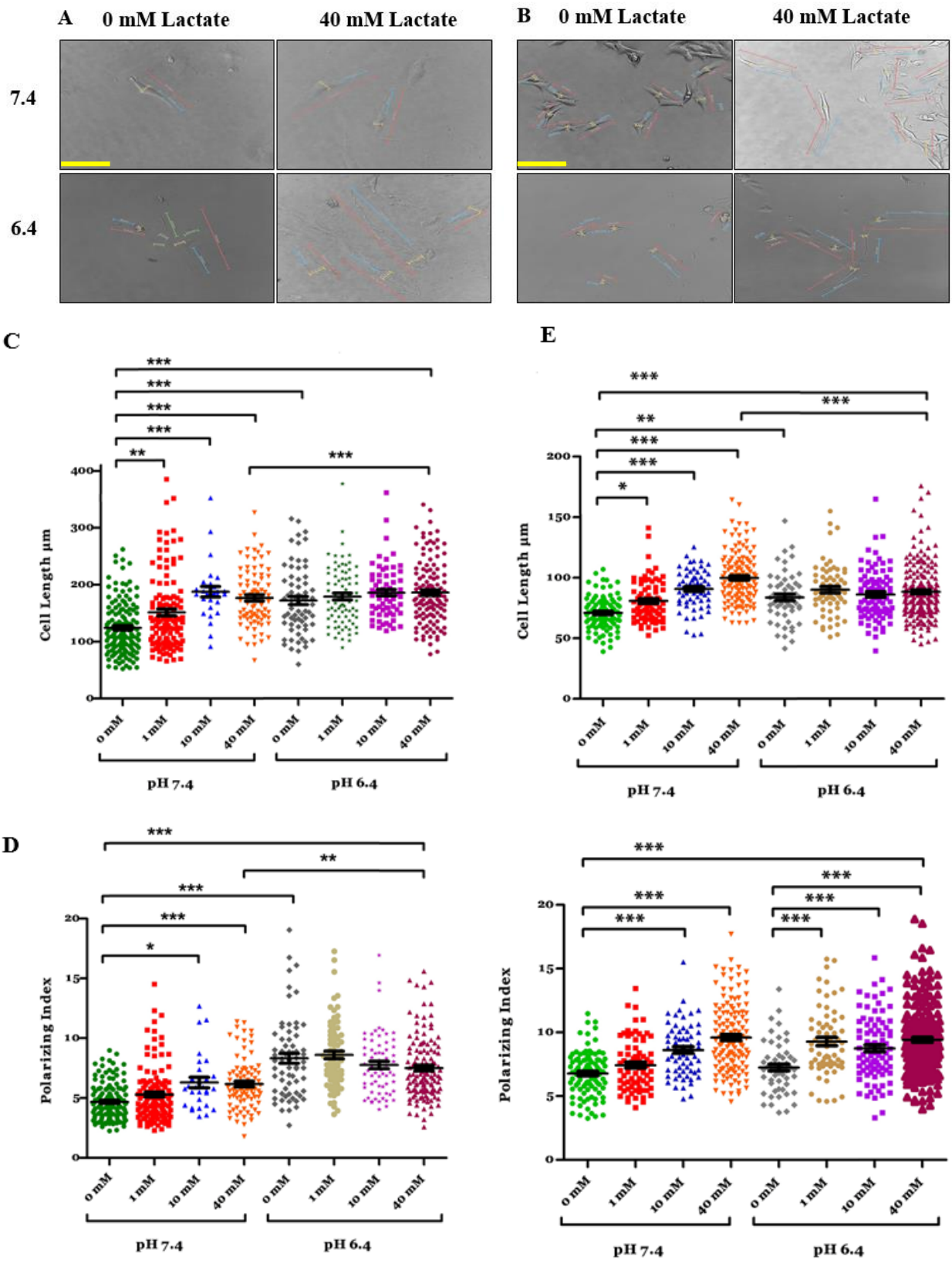


Figure 3.4. Cell actin staining after a wound assay experiment. **(A) HUVECs** were treated with EGM-2 buffered to pH 6.4 (w 40 mM and w/o Lactate) or 7.4 (w 40 mM and w/o Lactate). **(B) B16F10** cells were treated with DMEM with 10%FBS and 1% Penicillin-Streptomycin, buffered to pH 6.4 (w 40 mM and w/o Lactate) or 7.4 (w 40 mM and w/o Lactate). For Qualitative analysis, around 8-10 images were taken in each treatment group in each trial. ECHO revolve microscope at objective of 20x were used to capture the images. The scale bar for all images is 90µm.

3.5 Both cell models exhibited changes in morphology when exposed to acidic buffered media. The acidic treatment caused more actin stress formation in both cell models.

Two different buffered media were used to treat the cells: pH 7.4 (0 mM, and 40 mM Lactate) and pH 6.4 (0 mM, and 40 mM Lactate). Acidity was observed to cause cells became more elongated (**Figure 3.5 A, B, C, E**) with a higher polarizing index (**Figure 3.5 D, F**) due to difficulty in tail detachment, with tail separation from the cell body in HUVEC (**Figure 3.6 A, B**). When lactate was added to a pH 7.4 buffer, it significantly increased cell length in both cell types. However, acidity and lactate combined effect had an insignificant effect on cell length compared to acidic treatment only. It was observed that acidosis caused the accumulation of actin stress fibers and pFAK aggregates, particularly at the tip of the cell's tail, which further anchors the cell in its elongated shape (**Figure 3.6 C**).

Regarding the morphology of the two cell types, it's interesting to note that only the HUVECs, but not melanoma cells, produced multiple leading edges, or "Lamellipodia," when exposed to an acidic buffer (**Figure 3.7 A**). Additionally, under the combined action of lactate and acidity, the percentage of HUVECs with multiple leading edges dropped and aligned closer to the levels observed with physiological pH (**Figure 3.7 B**). This implies that lactate buffers the acidic effects on the migration-related morphology of HUVECs. Cells with multiple leading edges tend to change the migration behavior and migration mode of the cell[140,141]. In both cell models, more actin stress fibers were observed when cells were treated with acidic buffered media, which increased cytoskeletal tension that arises from excessive polymerization of actin filaments creating more stress fibers and contractile forces within the cell, leading to reduced cell migration rate. (**Figure 3.7C, D**).


A
0 mM Lactate
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7.4
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40 mM Lactate
C
Cell Length µm
0 mM
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pH 6.4
D
Polarizing Index
E
Cell Length µm
Polarizing Index

Figure 3.5. Single-cell morphology and actin staining **(A)** Representative images show the effect on cell length when **HUVECs** were incubated in 6.4 (0 mM and 40 mM lactate) compared to 7.4 (0 mM and 40 mM lactate) buffered media. Images were taken using the ECHO Revolve microscope at 20X objective bright field light. Scale bar is 110µm. **(B)** Representative images show the effect on cell length when **B16F10** cells were incubated in 6.4 (0 mM and 40 mM lactate) compared to 7.4 (0 mM and 40 mM lactate) buffered media. Images were taken using the ECHO Revolve microscope at 20X objective at bright field light. **(C)** scatter plotting represents the changes for HUVECs length when treated with 6.4 (0 mM,1mM, 10 mM, and 40 mM lactate) compared to 7.4 (0 mM,1 mM, 10 mM, and 40mM lactate) buffered media. **(D)** scatter plotting represents the polarizing index for HUVECs when treated with 6.4 (0 mM,1mM, 10 mM, and 40 mM lactate) compared to 7.4 (0 mM,1 mM, 10 mM, and 40 mM lactate) buffered media. **(E)** scatter plotting represents the changes for B16F10 cell length when treated with 6.4 (0 mM,1mM, 10 mM, and 40 mM lactate) compared to 7.4 (0 mM,1 mM, 10 mM, and 40mM lactate) buffered media. **(F)** scatter plotting represents the polarizing index for B16F10 when treated with 6.4 (0 mM,1mM, 10 mM, and 40 mM lactate) compared to 7.4 (0 mM,1 mM, 10 mM, and 40 mM lactate) buffered media. Each scatter blot in the graph represents the average of 3 trials (n=3). The statistical significance was calculated using one-way ANOVA followed by Tukey post-test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. The scale bar is 100µm.

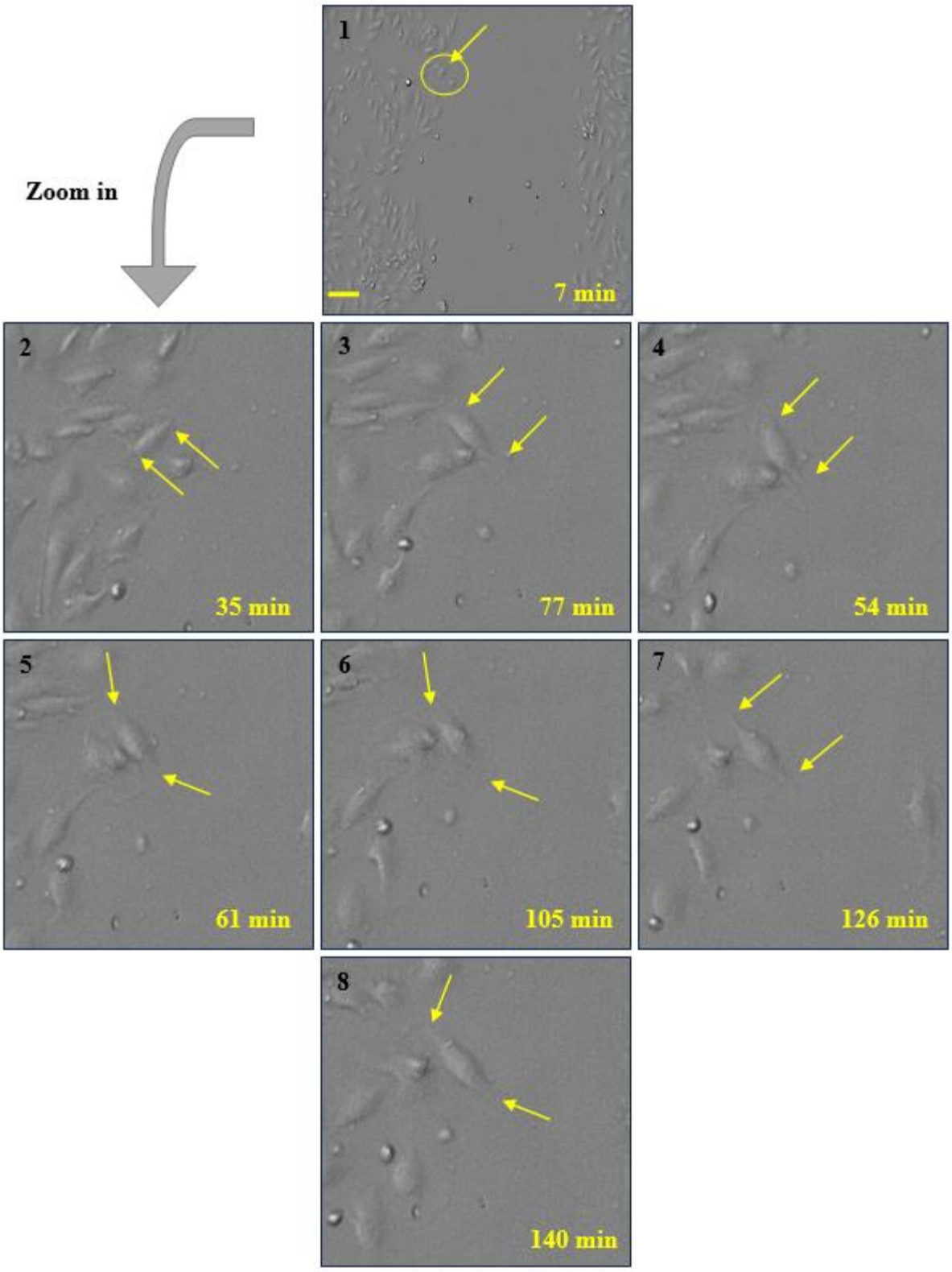

Figure 3.6 A. A demonstration of normal HUVEC migration steps under a physiological pH environment. The cells exhibit organized attachment at the leading edge, cell spreading, followed by a smooth detachment and forward retraction. Images were extracted from a time-lapse video using the CD7 microscope from Zeiss, at lens objective of 5x was used to capture the images. The scale bar is 100 µm.

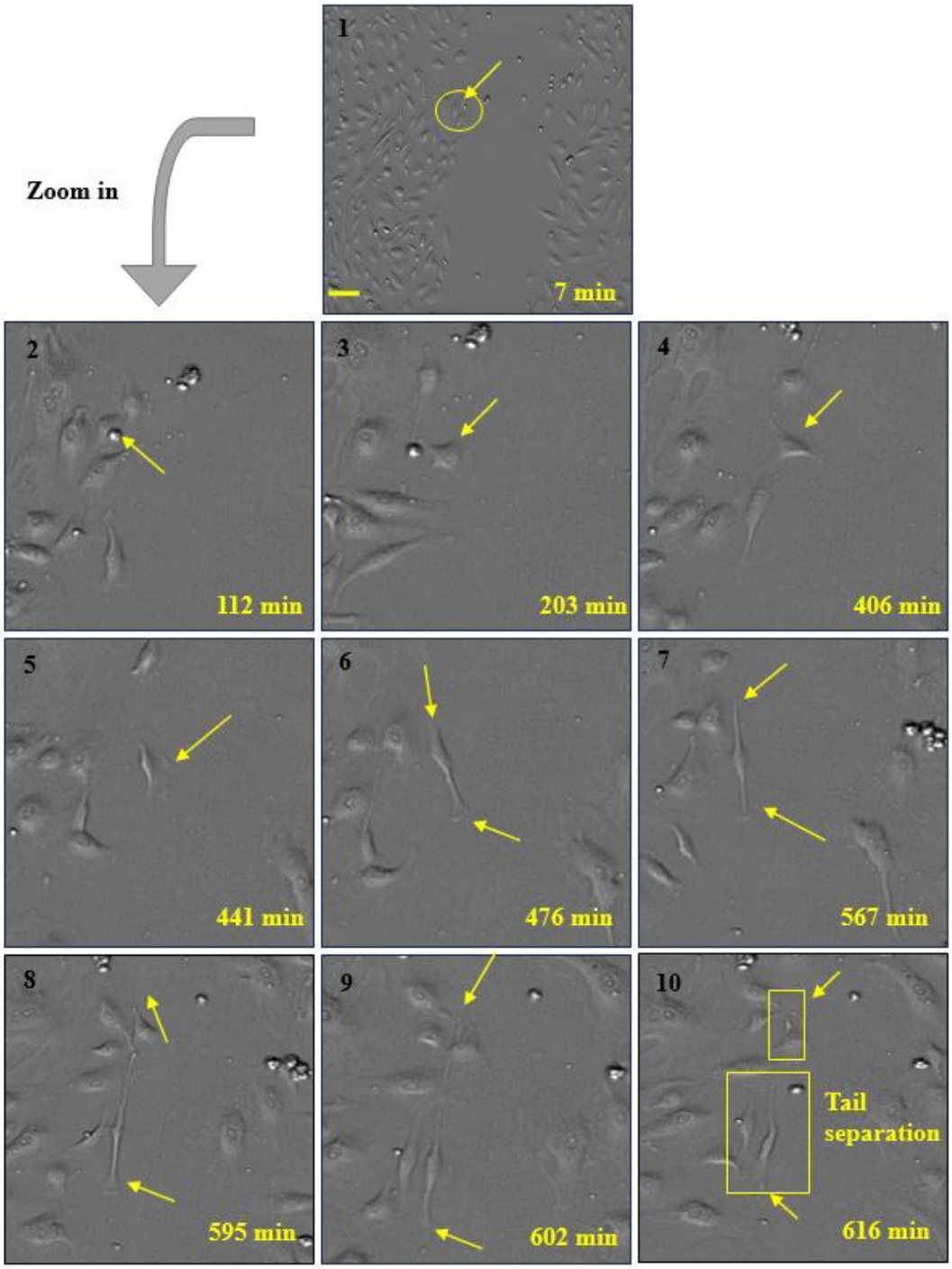

Figure 3.6 B. HUVECs exhibited changes in morphology when exposed to acidic buffered media. The acidity was observed to cause cells became more elongated due to difficulty in tail detachment, with tail separation from the cell body in HUVECs. Images were extracted from a time-lapse video using the CD7 microscope from Zeiss, at lens objective of 5x was used to capture the images. The scale bar is 100 μm.

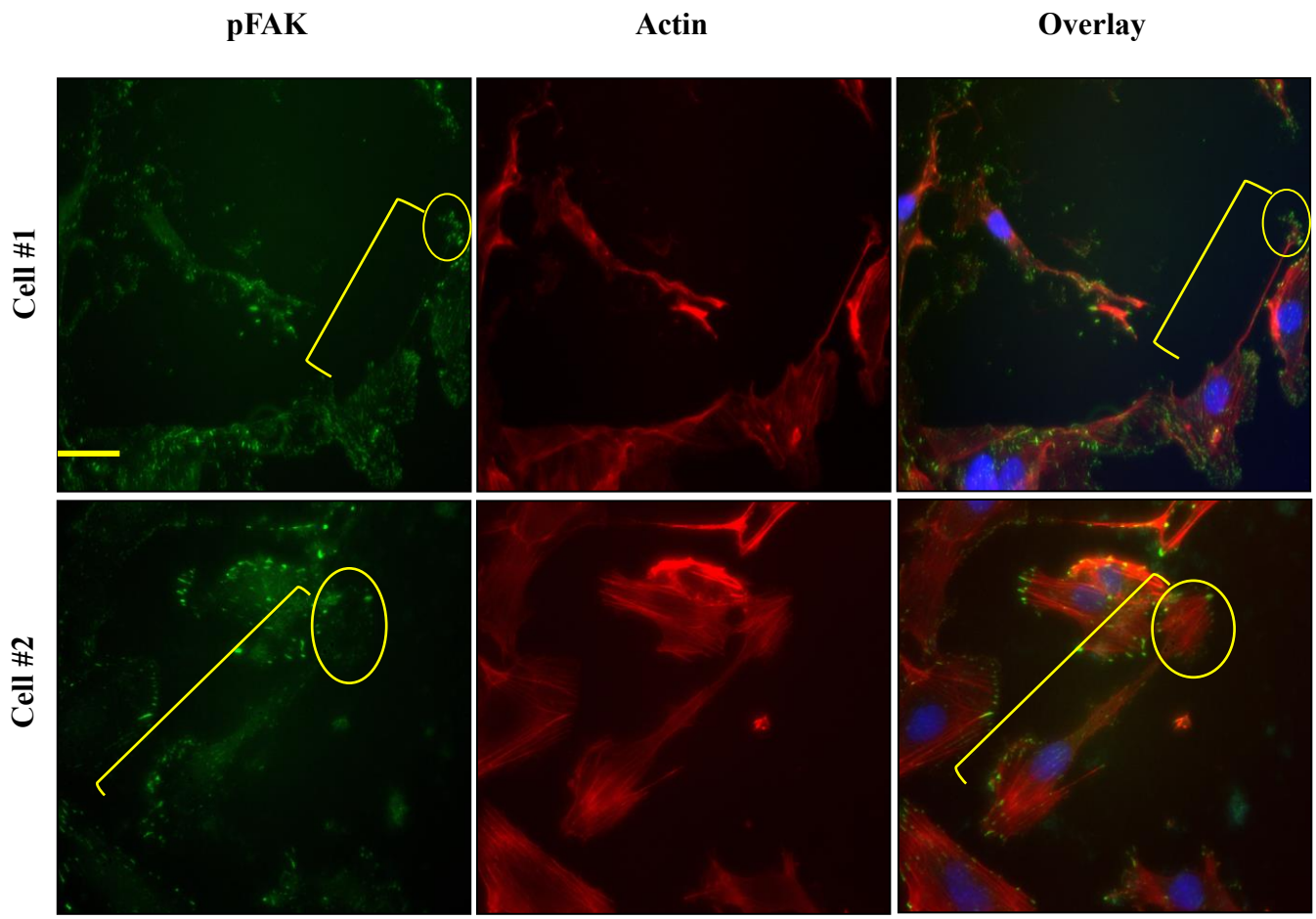

Figure 3.6 C. Accumulation of pFAK and actin stress fibers in the tip of the HUVECs tail during migration. this suggests that the acidity was observed to cause cells became more elongated due to difficulty in tail detachment, with tail separation from the cell body in HUVECs. ECHO revolve microscope at an objective of 40x was used to capture the images. The scale bar is 50 µm.

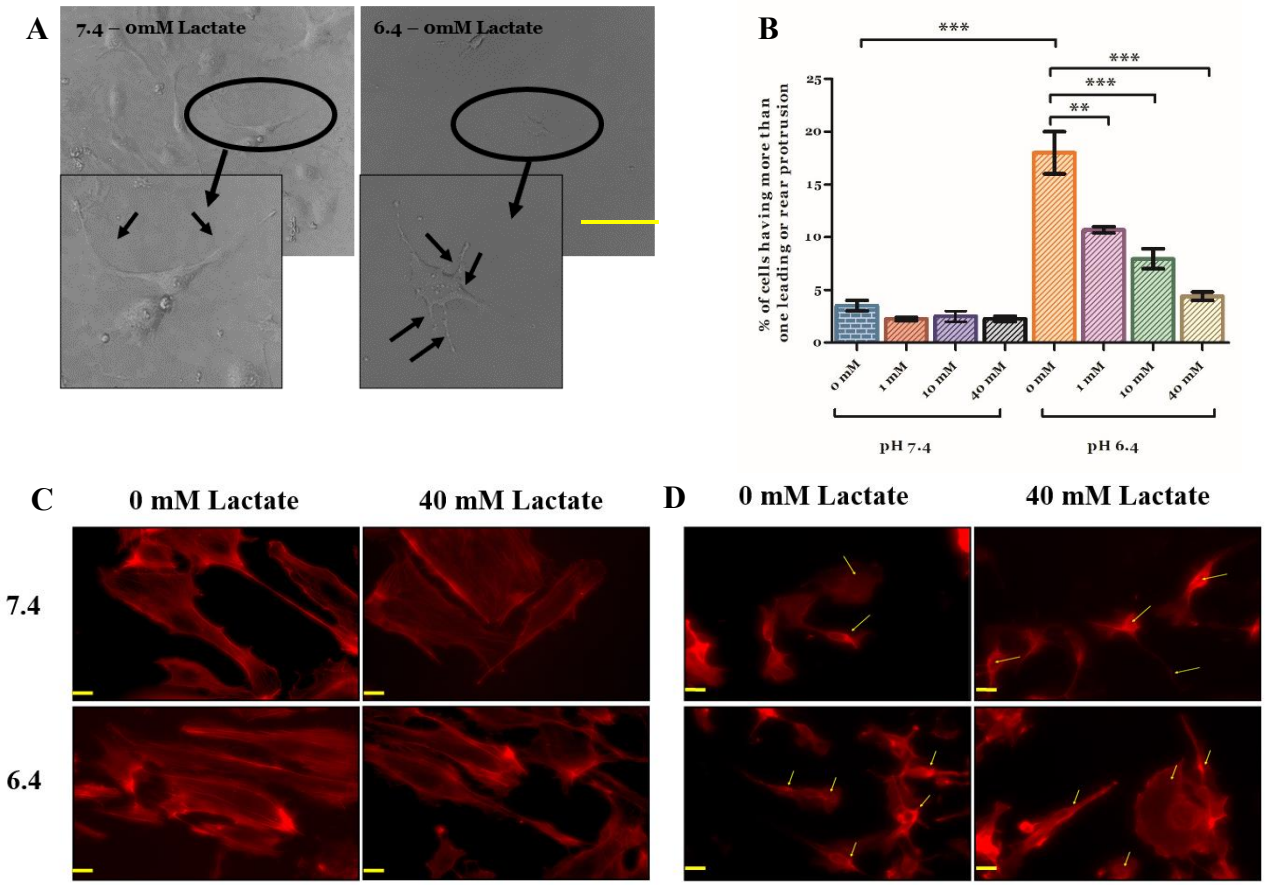

Figure 3.7. **(A)** Representative (cropped) images show the increase of multiple protrusions in HUVEC cells when incubated in 6.4 (0 mM lactate) compared to 7.4 (0 mM lactate) buffered mediums. Images were taken using the ECHO Revolve microscope with 20X objective at bright field light. Scale bar = 110 µm. **(B)** bar-graph demonstrating the increase of multiple protrusions in **HUVEC** cells when incubated in 6.4 (0 mM and 40 mM lactate) compared to 7.4 (0 mM lactate) buffered mediums. Each bar in the graph represents the average of 3 trials (n=3). The statistical significance was calculated using one-way ANOVA followed by Tukey post-test. *P < 0.05, **P < 0.01, and ***P < 0.001. **(C)** Cell actin staining for HUVECs treated with EGM-2 buffered to pH 6.4 (w 40 mM and w/o Lactate) or 7.4 (w 40 mM and w/o Lactate) in single-cell morphology assay **(D)** Cell actin staining for B16F10 treated with DMEM with 10% FBS buffered to pH 6.4 (w 40 mM and w/o Lactate) or 7.4 (w 40 mM and w/o Lactate) in single-cell morphology assay. For Qualitative analysis, around 15 images were taken in each treatment group in each trial. ECHO revolve microscope at an objective of 20x was used to capture the images. The scale bar is 90 µm.

3.6 Acidity delayed HUVEC and B16F10 cell attachment and spreading, while the combination effect of acidity and lactate reduces the negative effects of acidity.

Taking the investigation in this project a step further, as the cell attachment is vital in initiating signaling pathways that promote spreading and stabilize the cell and prepare it for migration, A single-cell attachment assay was performed on both HUVEC and melanoma cells. The analyzed qualitative data showed that HUVEC (**Figure 3.8A, 3.7C**) and B16F10 cell (**Figure 3.8E, 3.7G**) attachment was delayed when plated in media buffered to pH 6.4 compared to pH 7.4, with an increase of 29% in HUVEC round cells (**Figure 3.9A**), and 37% in B16F10 round cells (**Figure 3.9D**) as an indication of attachment delay under acidic treatment compared to physiological one. Furthermore, it was observed in both cell models that lactate altered these effects by reducing the negative impact of acidity on both HUVEC (**Figure 3.8C, 3.7D**) and B16F10(**Figure 3.8G, 3.7H**) attachment, with a 10% decrease in HUVEC round cells (**Figure 3.9A**) and a 29 % decrease in B16F10 round cells (**Figure 3.9D**) when cells were treated with a combined buffer of acidity and lactate compared to acidic treatment only. Next, we investigated the effect of acidity and lactate upon the spreading of both cell models, HUVEC and B16F10 cell spreading was delayed when plated in media buffered to pH 6.4 compared to pH 7.4, with a 10 % decrease in HUVEC (**Figure 3.9B**) attached cell, and 30 % decrease in B16F10 attached cells (**Figure 3.9E**). it was observed that attachment was enhanced when cells were treated with acidity and high lactate levels combined, with a 14% increase in HUVEC spreading cells (**Figure 3.9B**) and a 33 % increase in B16F10 spreading cells (**Figure 3.9E**) compared to acidic treatment without lactate. Lastly, when it comes to polarized cells, acidity decreased the polarized cells in both models, with a 32% decrease in polarized HUVEC (**Figure 3.9C**) and 67% decrease in B16F10 polarized cells (**Figure**

3.9F), which means that acidity blocked the transition of spreading cells to the migrating phase. Moreover, the combination effect of acidity and lactate showed a further reduction of the percentage of polarized cells with a 44% decrease in HUVEC polarized cells (**Figure 3.9C**) and a 26% decrease in B16F10 polarized cells (**Figure 3.9F**) compared to acidic treatment only.

Because acidity influences the formation of F-actin in cells[142], HUVEC and B16F10 cells were stained for F-actin. In both cell models (round cells), actin showed an altered actin arrangement _actin aggregate at one side of the cell_ when cells were treated with acidic buffer, and when treated with acidic buffer and lactate combined (**Figure 3.10A**). the alteration of actin arrangement in a spreading cell was more obvious in HUVEC than B16F10 (**Figure 3.10B**). at pH 7.4 more uniform distribution of actin throughout the cytoplasm compared to cells treated with pH 6.4 buffer, and the actin aggregates and formation of actin fiber at the cell peripheral was more obvious when lactate added to the acidic treatment.

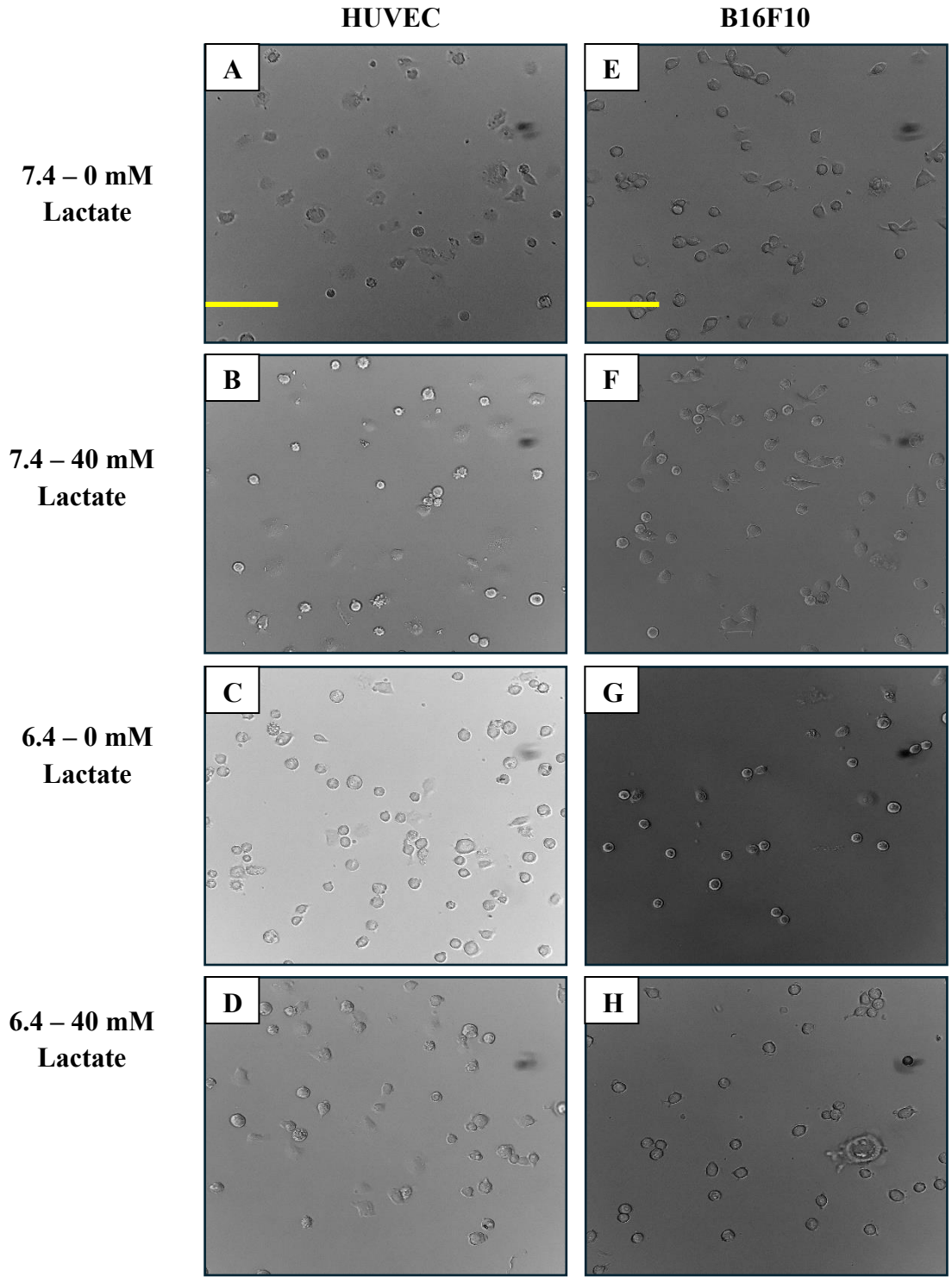

Figure 3.8: Representative images of HUVEC and B16F10 Cell Spreading after 1 hour of single cell attachment assay **(A)** HUVEC cell attachment and spreading at pH 7.4. **(B)** HUVEC cell attachment and spreading at pH 7.4 with 40 mM Lactate. **(C)** HUVEC cell attachment and spreading at pH 6.4. **(D)** HUVEC cell attachment and spreading at pH 6.4 with 40 mM Lactate. **(E)** B16F10 cell attachment and spreading at pH 7.4. **(F)** B16F10 cell attachment and spreading at pH 7.4 with 40 mM Lactate. **(G)** B16F10 cell attachment and spreading at pH 6.4. **(H)** B16F10 cell attachment and spreading at pH 7.4 with 40 mM Lactate. The assay was repeated three times using an ECHO revolved microscope at 20X objective (110 µm scale bar).

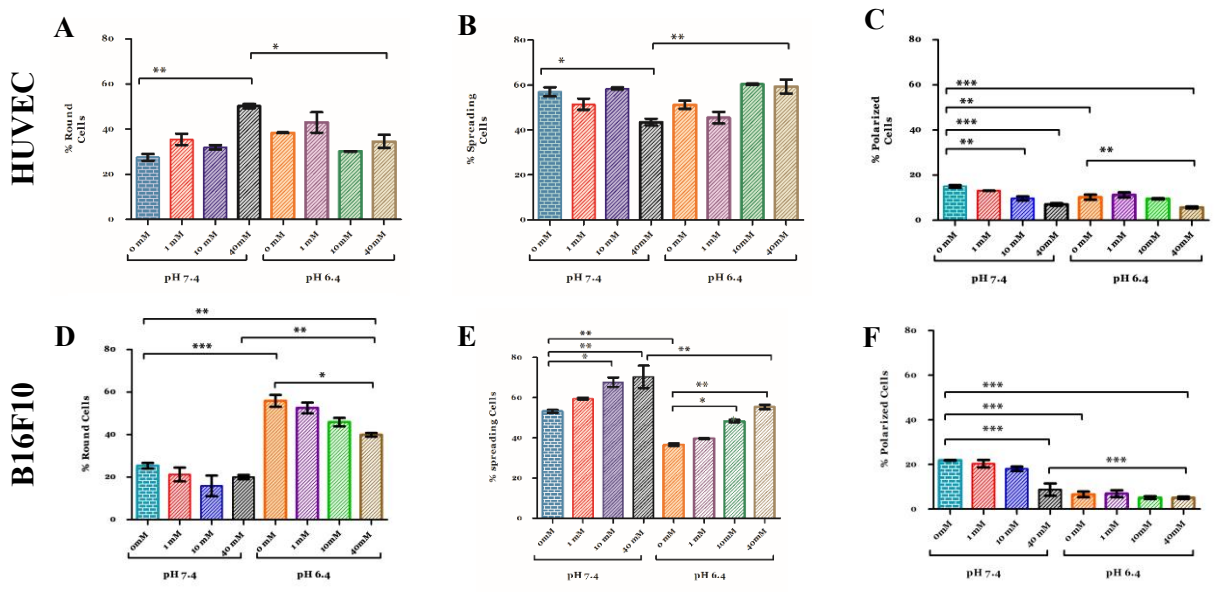

Figure 3.9: Quantitative analysis for the effect of acidity and lactate on delaying the cell attachment and spreading (**A) and (D)** the percentage of round cells when treated with various acidity and lactate levels. (**B)** and **(E)** the percentage of spreading cells when treated with various acidity and lactate levels. **(C)** and **(F)** the percentage of Polarized cells when treated with various acidity and lactate levels. Each bar in the graph represents the average of 4 different biological trials (n=4). Number of cells analyzed and counted: **B16F10** pH 6.4 = 2410 Cells, pH 7.4 = 1997 Cells. **HUVEC** pH 6.4 = 1361 Cells, pH 7.4 = 1099 Cells. The average percentages were tested for statistical significance using one-way ANOVA followed by Newman-Keuls multiple comparison test using GraphPad Prism 5 software. *P < 0.05, **P < 0.01, and ***P < 0.001, compared to control.

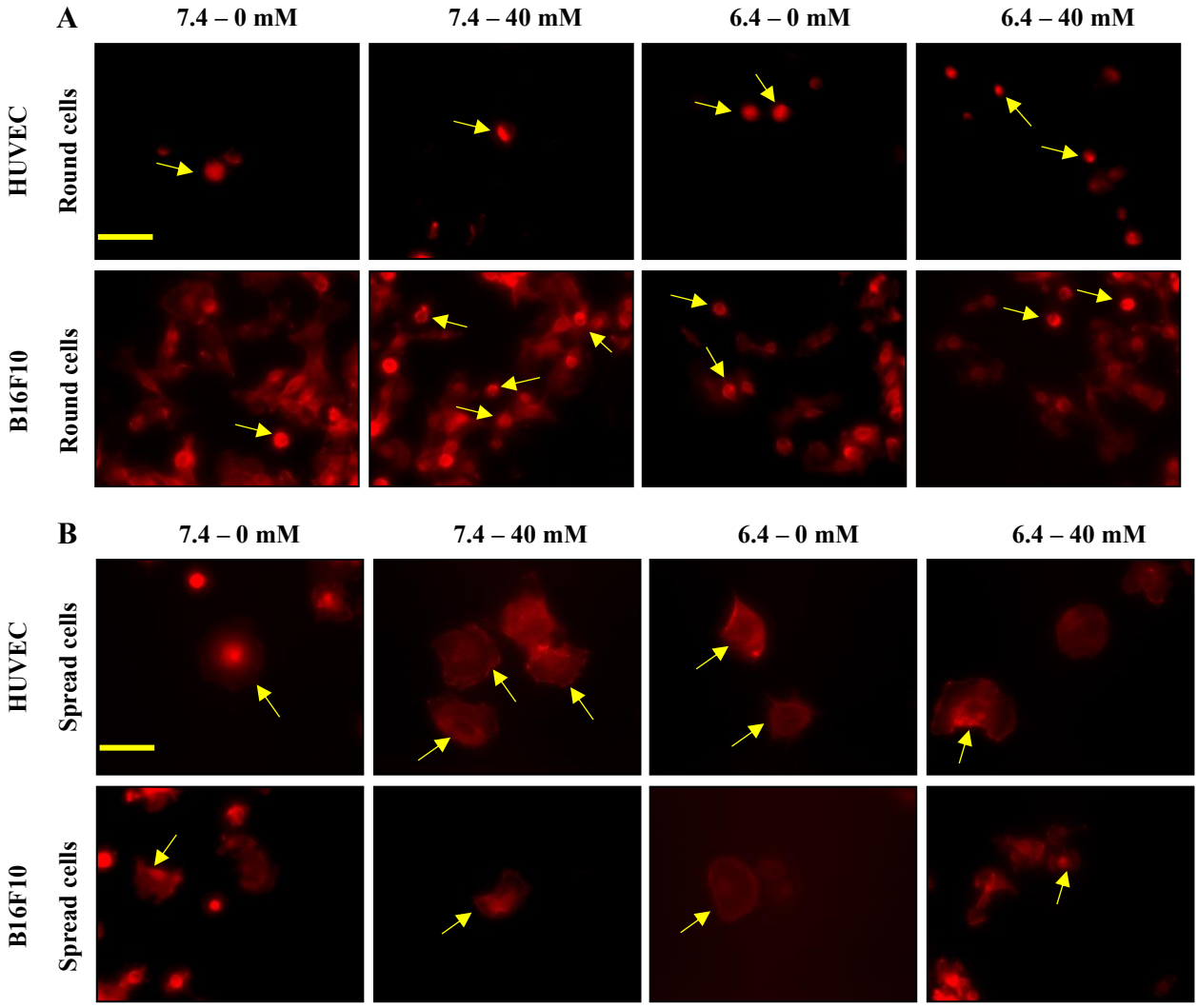

Figure 3.10: The effect of acidity and lactate on cell attachment for 1 h experiment on actin stress fibers formation **(A)** actin formation in round HUVEC and B16F10 cells treated with different pH buffers with and without lactate. **(B)** actin formation in spread HUVEC and B16F10 cells treated with different pH buffers with and without lactate. The assay was repeated three times using an ECHO revolved microscope at 40X objective. The scale bar is 50 µm.

3.7 The localization of phospho-focal adhesion kinase (Y397) altered by Acidity and acidity combined with lactate in HUVEC and B16F10 cell migration, attachment, and spreading.

Focal adhesions are critical in cell migration, attachment, and spreading. These dynamic structures are essential for establishing the main contact points between a cell and the extracellular matrix (ECM). The positioning of focal adhesions allows for localized signaling that promotes stable attachment, spreading, and migration for cells.[143,144] So based on that, we investigated the alteration in focal adhesion localization and dynamics during cell migration, attachment, and spreading for both HUVEC and B16F10 cells when treated with acidic media with and without lactate. Immunocytochemistry (ICC) was used to stain for the focal adhesion kinase and phosphor paxillin proteins, as those proteins are crucial for the complex processes of cell attachment, spreading, and migration. Their functions are interdependent and critical for maintaining cellular dynamics and tissue integrity. The focal adhesions are indicated by the localization of pFAK (Y397) and pPAX (Y118). Cell movement, signaling, and adhesion all depend on these complexes, their activation level and their locations.[145] The localization of phospho-focal adhesion kinase was investigated in migrated, attached, and spread cells in both HUVEC and B16F10 were investigated. For both cell models, it was observed that the location of pFAK (Y397) was altered when cells were treated with a pH 6.4 buffered medium, pFAK (Y397) is mainly found at the outer boundary of the cell (cell periphery). This positioning is linked to the heightened formation of focal adhesions at the edges of the cell. The treatment of HUVEC cells with pH 6.4 and 40 mM lactate results in activation of FAK in central regions besides the cell periphery. This suggests alterations in focal adhesion dynamics and actin cytoskeleton organization in response to the combined influence of acidity and lactate. The comparable impact on PFAK (Y397) localization

seen when using 40 mM lactate in a pH 7.4 buffer, as opposed to acidic conditions, may be credited to lactate's ability to prompt metabolic and signaling alterations in the cells. These alterations alter the cellular reaction to acidic stress, leading to pFAK (Y397) activation and the rearrangement of focal adhesions and actin stress fibers, besides clustering of pFAK (Y397) was observed to be bigger and higher when cells were treated with acidic buffer **(Figure 3.11)** and **(Figure 3.12)**. An additional step in the investigation included examining the alterations in pFAK (Y397) distribution as cell morphology (cell shape and elongation) changed in reaction to different pH treatments with and without lactate through the execution of a single cell migration assay **(Figure 3.13)** and **(Figure 3.14)**. A distinct elongation of HUVEC and B16F10 cells in acidic environments and with lactate was observed when studying the effect of acidity on the cells when a single-cell migration assay was carried out to study the changes in cell morphology in both cell models. Studying the pFAK (Y397) and actin fibers, actin stress fibers formed under acidic treatment in both cell models but with greater significance in HUVECs compared to B16F10. When it comes to the pFAK (Y397) location, under mildly acidic conditions (pH 6.4), pFAK (Y397) is primarily found at the periphery of the cells in both cell models, with a more significant presence in HUVEC cells, while pFAK (Y397) was in both cell periphery and cell body when cells were treated with pH 7.4 buffered media. This localization correlates with enhanced focal adhesion formation at the cell margins, facilitating the stabilization of cell attachment to the extracellular matrix (ECM) during stress conditions, such as low pH. A similar effect was noted when cells were exposed to an acidic buffer and 40 mM lactate, reducing aggregated pFAK (Y397). The same phenomenon was observed when cells were treated with 40mM lactate in a pH 7.4 buffer mimicking the same effect when cells were treated with acidic treatment.

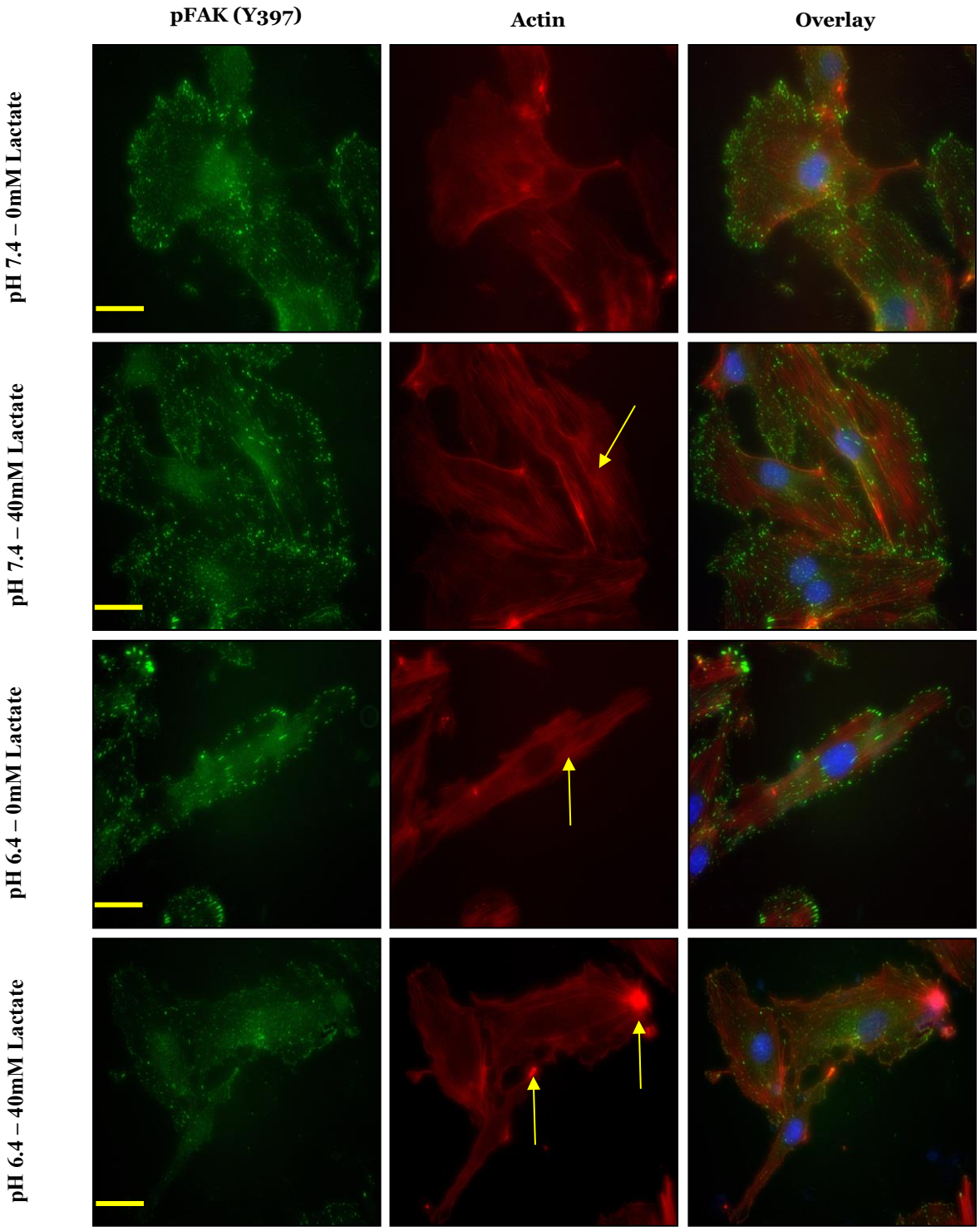

Figure 3.11: Representative images for HUVEC at the edges of wound scratch for a 13h wound scratch assay. Cells were stained for pFAK (Y397) localization when cells treated with acidic buffer with/without lactate by Immunocytochemistry (ICC). Four different biological trials (n=4) were carried out. Images were taken using an ECHO revolved microscope at 40X objective. The scale bar is 50 µm.

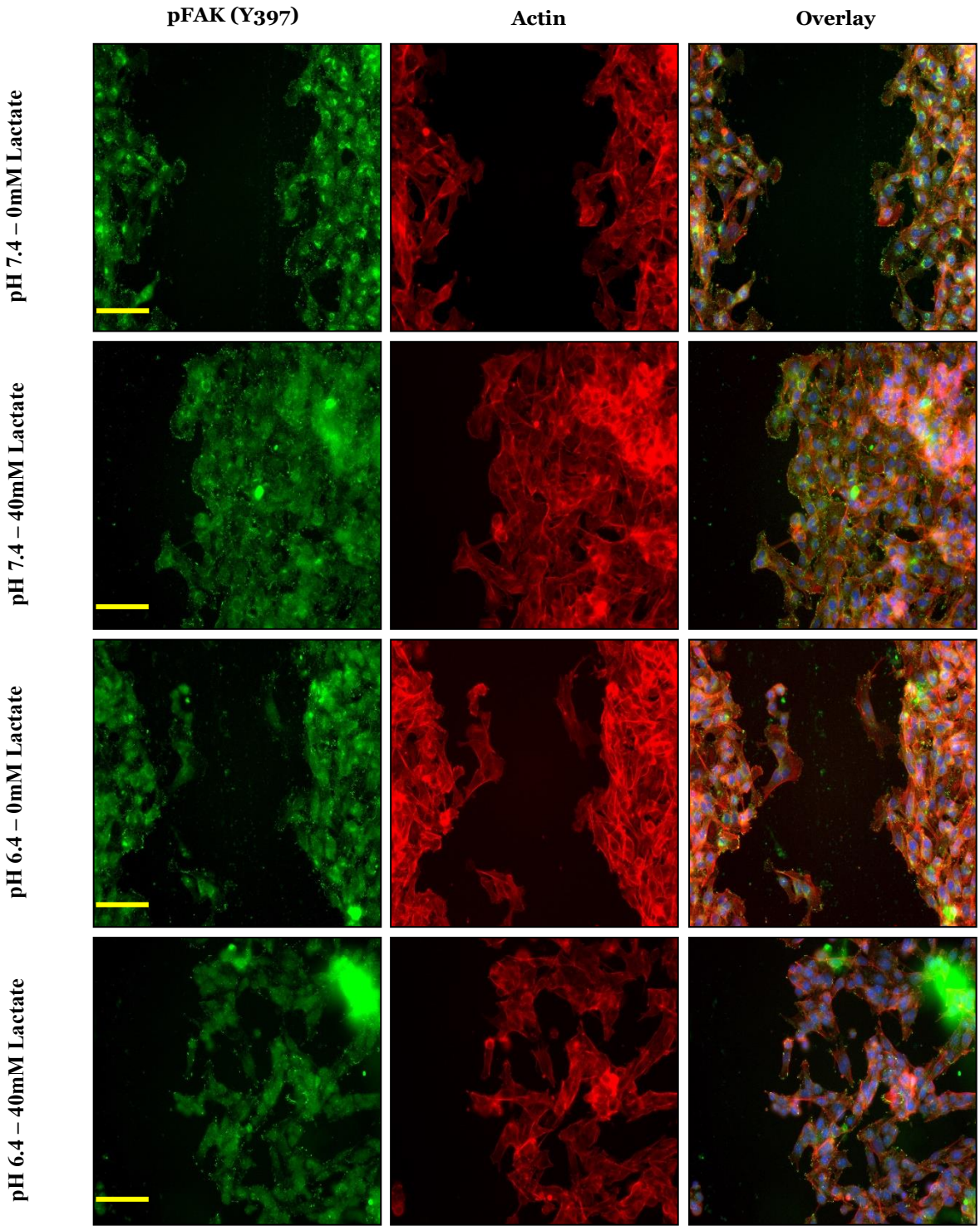

Figure 3.12: Representative images for B16F10 at the edges of wound scratch for an 11h wound scratch assay. Cells were stained for pFAK (Y397) localization when cells were treated with an acidic buffer with/without lactate by Immunocytochemistry (ICC). Four different biological trials (n=4) were carried out. Images were taken using an ECHO revolved microscope at 20X objective. The scale bar is 90 µm.

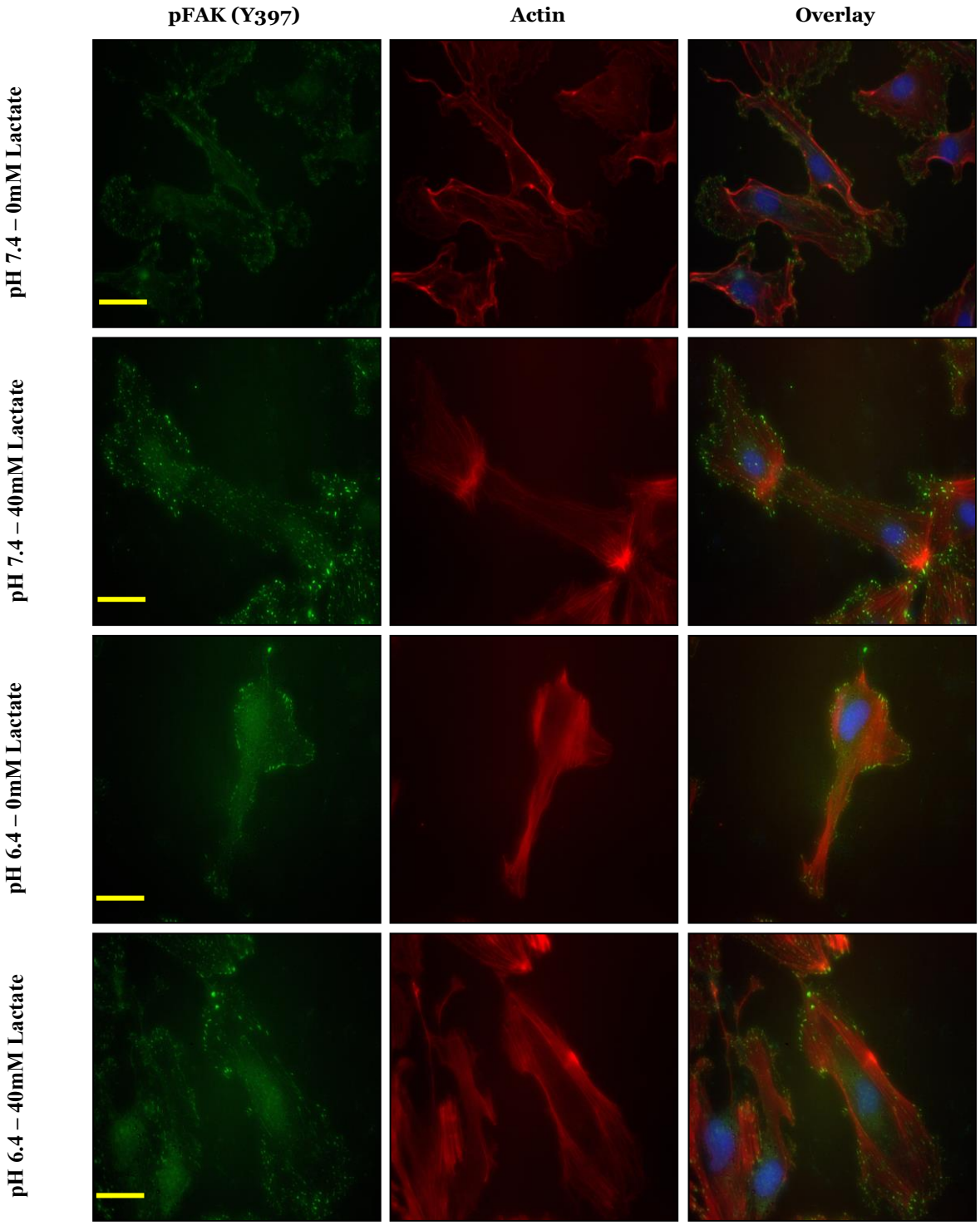

Figure 3.13: Representative images for HUVEC morphology via single cell migration assay. Cells stained for pFAK (Y397) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Four different biological trials (n=3) were carried out. Images were taken using an ECHO revolved microscope at 40X objective. The scale bar is 50 μm.

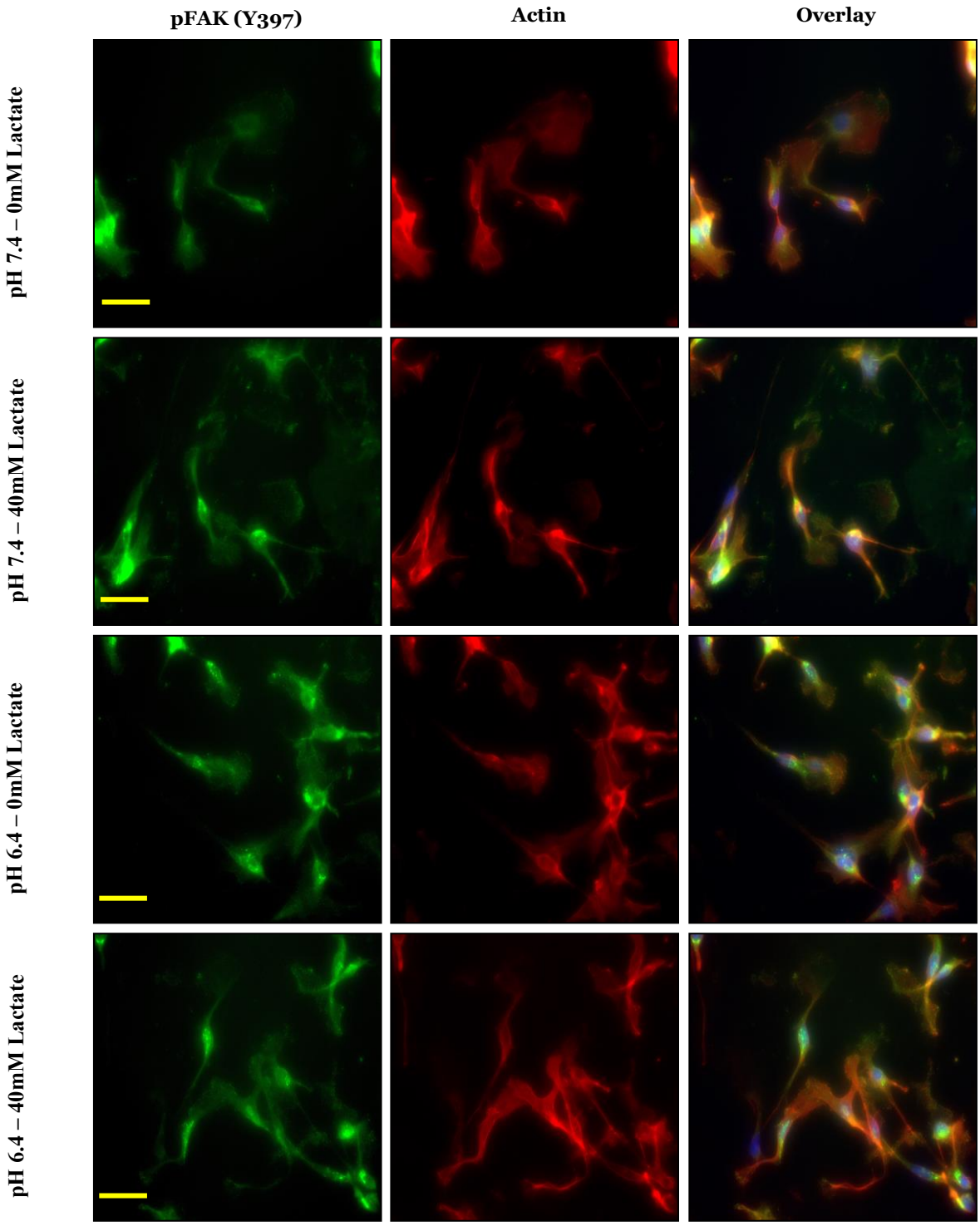

Figure 3.14: Representative images for B16F10 morphology via single cell migration assay. Cells stained for pFAK (Y397) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Four different biological trials (n=4) were carried out. Images were taken using an ECHO revolved microscope at 40X objective. The scale bar is 50 μm.

One last step was taken in investigating the localization of pFAK (Y397) alteration by different treatments, round attached and spread cells were stained for pFAK (Y397) in both HUVECs. In both round and attached cell models, acidity caused a change in phosphorylation locations in the cell or re-localization of pFAK (Y397) whether it's in round (barley attached) or spreading cells. It was seen that the phosphorylation of FAK was located both in the cell body and cell peripheral when cells were treated with pH 7.4 buffered media. At the same time, it was more located in the periphery when cells were under the influence of acidity. the combined effect of lactate and acidity was obvious in reducing phosphorylation locations. The lactate effect without acidity showed significant relocation of pFAK (Y397) and reduced phosphorylation spots **(Figure 3.15** and **Figure 3.16)**. In both spread cell models, acidity also showed a significant change in phosphorylation locations in the cell or re-localization of pFAK (Y397). The phosphorylation of FAK was observed to be present in both the cell body and the peripheral regions when cells were exposed to media buffered at pH 7.4. However, under acidic conditions, a greater concentration of phosphorylation was noted in the peripheral areas with a larger elongated size of pFAK (Y397). The interaction between lactate and acidity clearly demonstrated a reduction in the locations of phosphorylation besides the distribution of the FAK phosphorylated locations close to physiological conditions. Furthermore, the presence of lactate alone, in the absence of acidity, resulted in pFAK (Y397) distribution similar to acidic treatment in HUVEC, while in B16F10 the redistribution of pFAK (Y397) showed a decrease in phosphorylation sites. **(Figure 3.17** and **Figure 3.18)**.

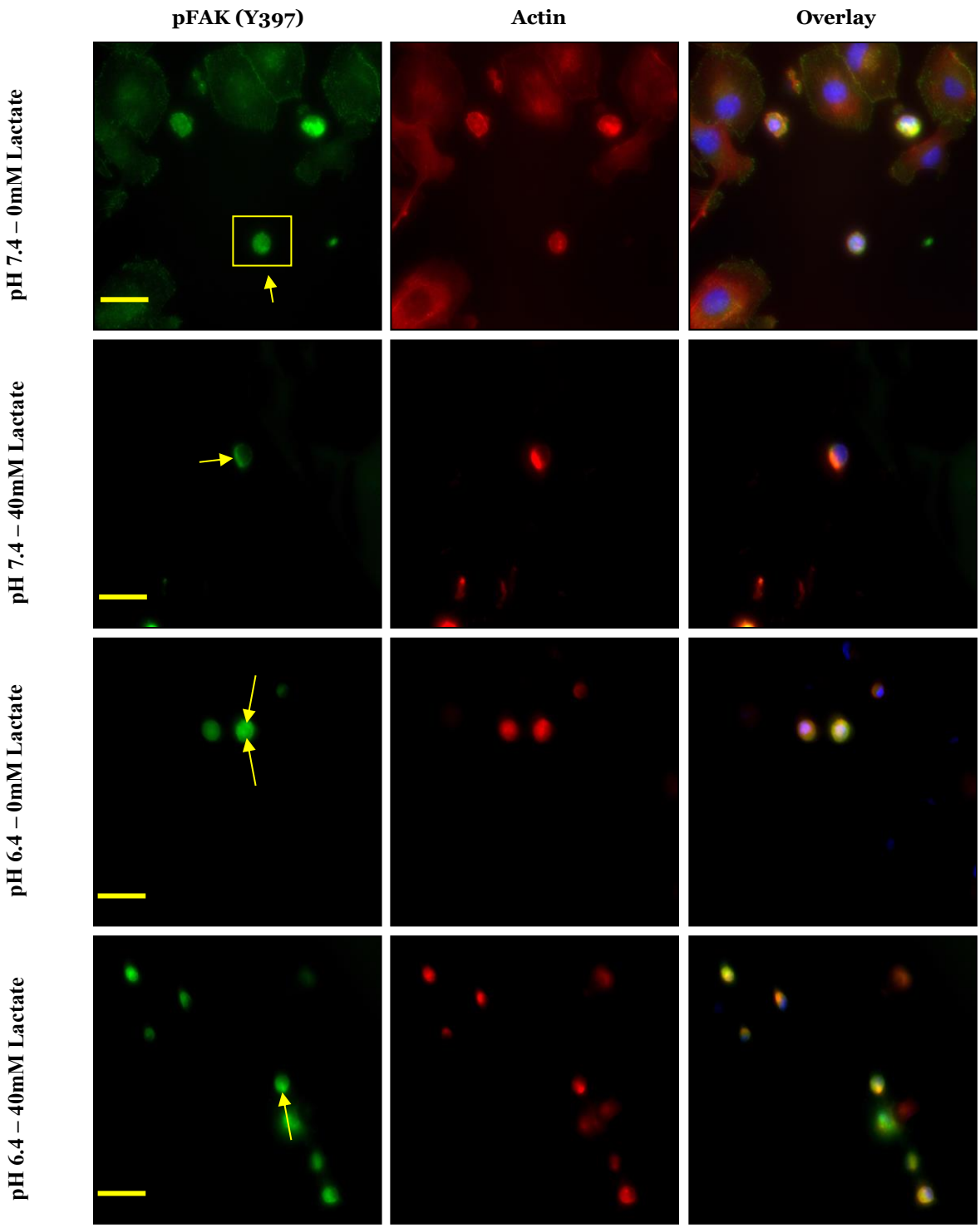

Figure 3.15: Representative images for ***round*** **HUVEC** via cell attachment assay. Cells stained for pFAK (Y397) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Four different biological trials (n=3) were carried out. Images were taken using an ECHO revolved microscope at 40X objective. The scale bar is 50 µm.

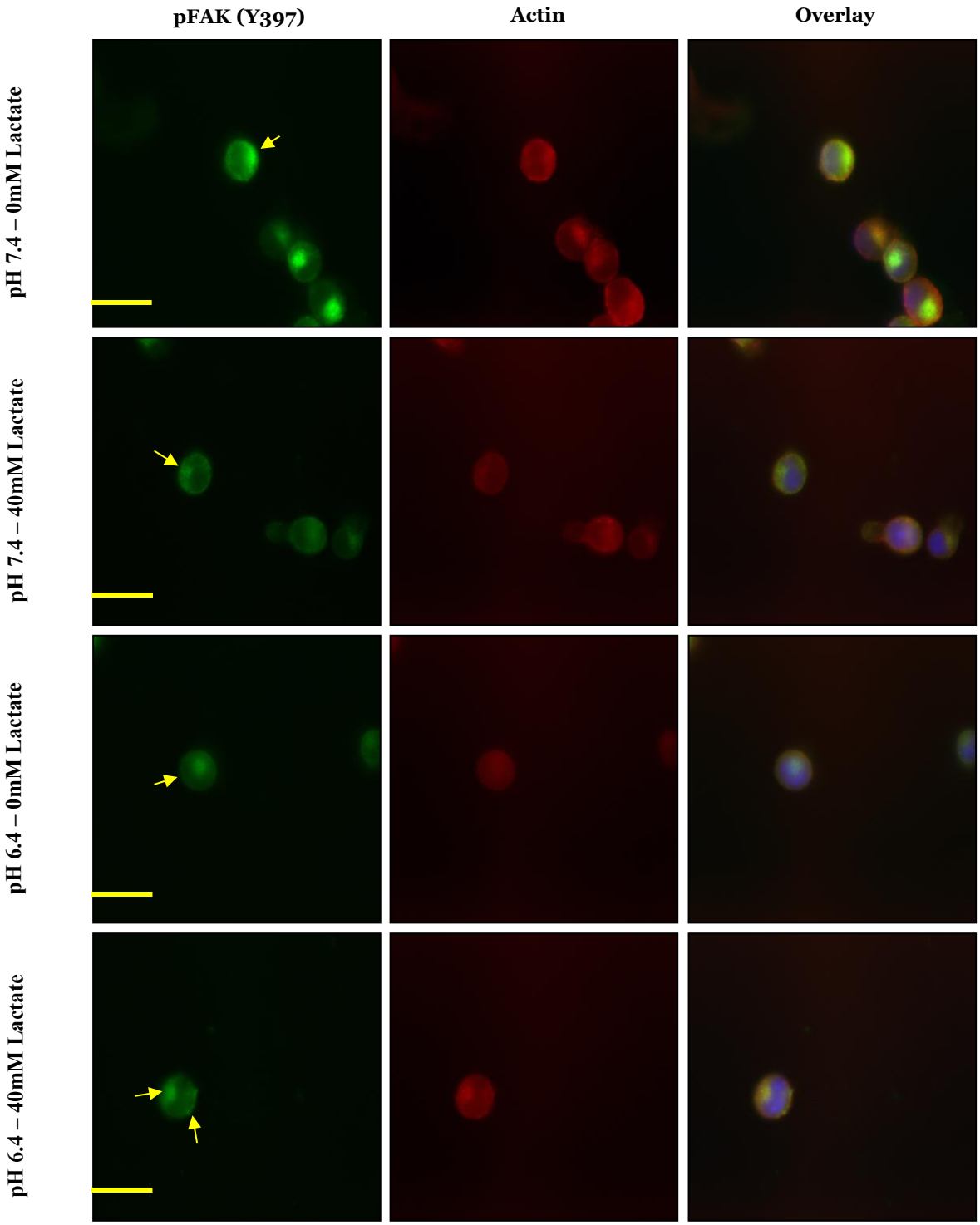

Figure 3.16: Representative images for ***round*** **B16F10** via cell attachment assay. Cells stained for pFAK (Y397) (Y397) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Noting that cells showed the production of multiple projections when treated with acidic buffered media. Three different biological trials (n=3) were carried out. Images were taken using an ECHO revolved microscope at 60X objective. The scale bar is 30 µm.

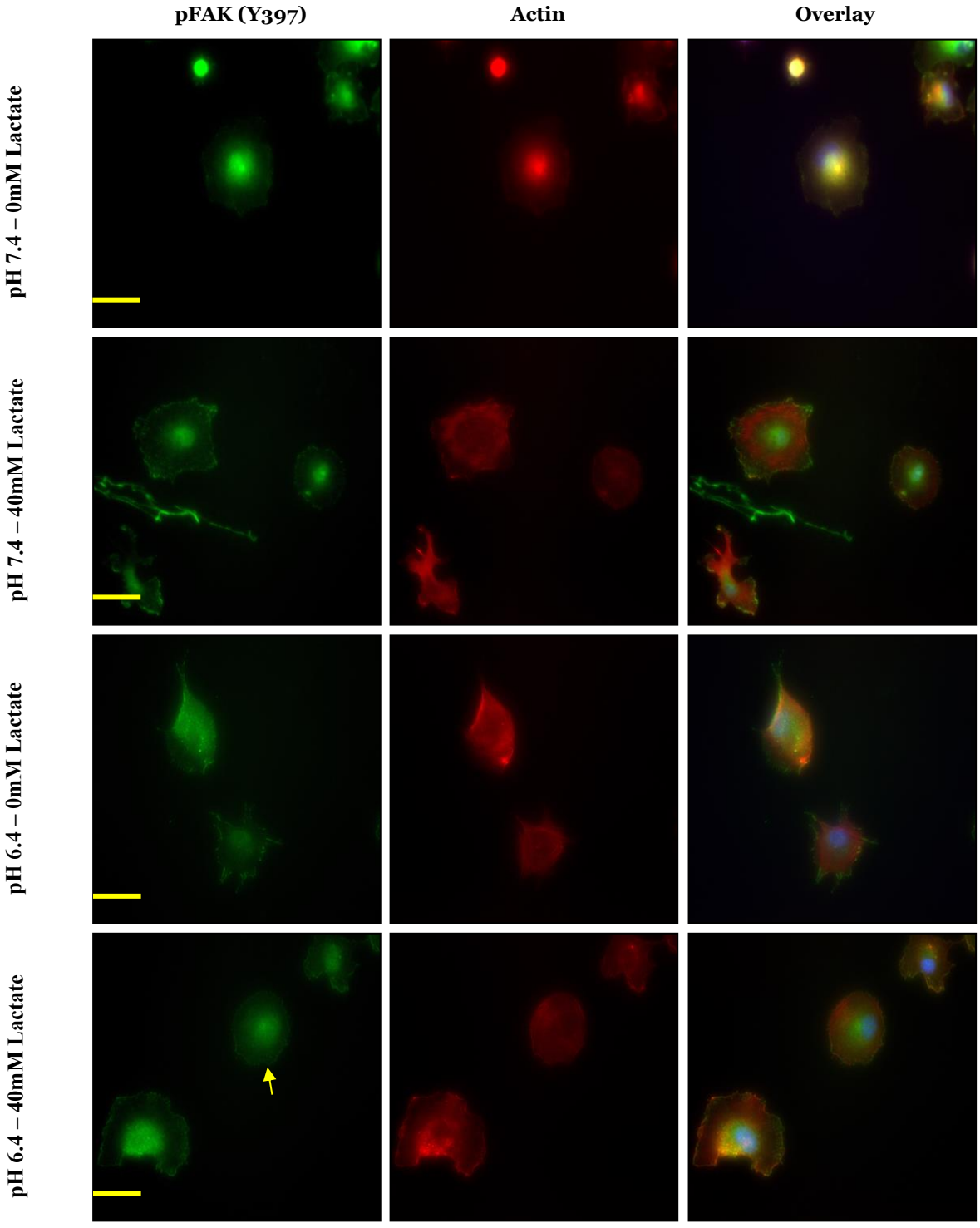

Figure 3.17: Representative images for ***Spread*** **HUVEC** via cell attachment assay. Cells stained for pFAK (Y397) (Y397) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Noting that cells showed the production of multiple projections when treated with acidic buffered media. Three different biological trials (n=3) were carried out. Images were taken using an ECHO revolved microscope at 40X objective. The scale bar is 50 µm.

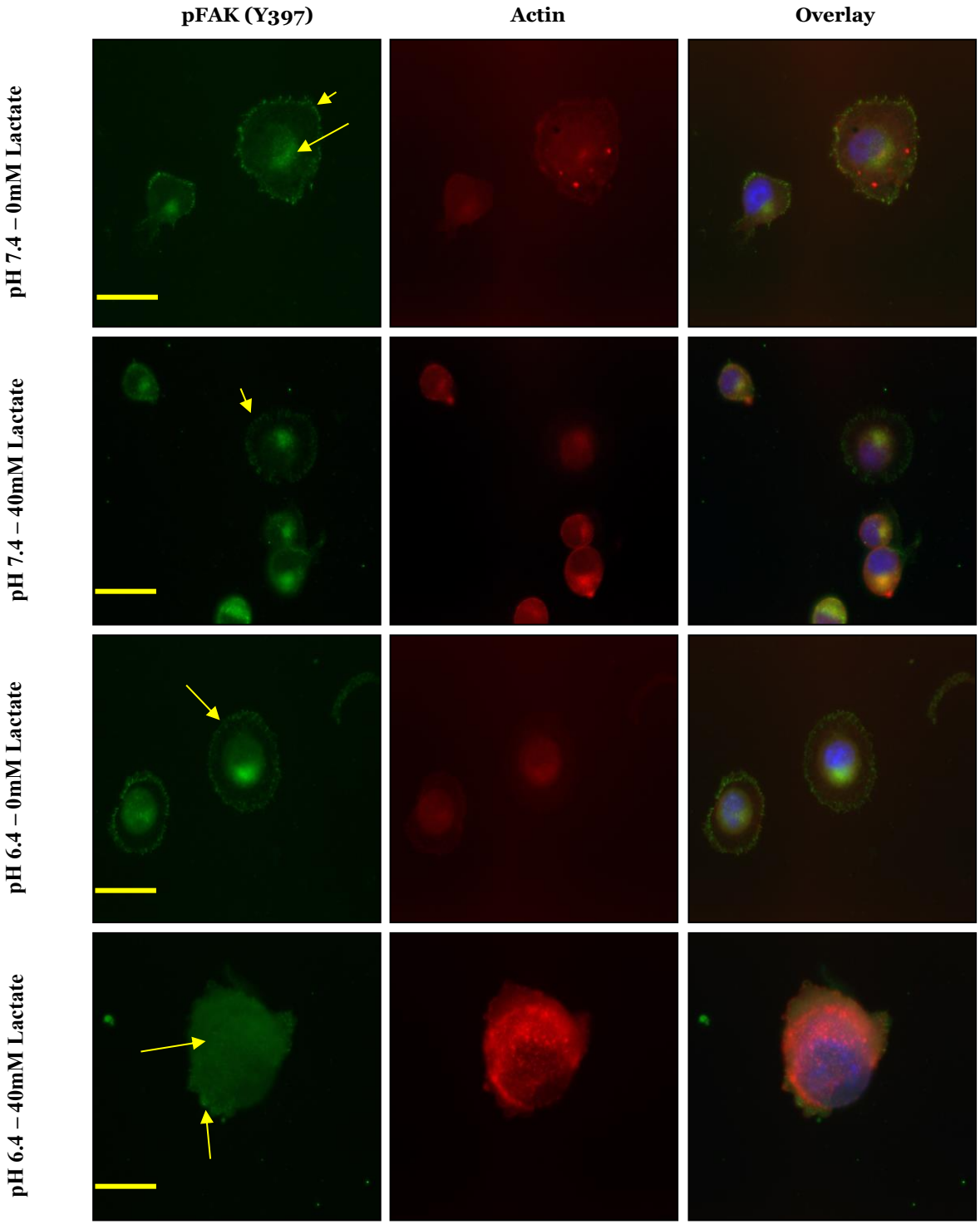

Figure 3.18: Representative images for ***Spread*** **B16F10** via cell attachment assay. Cells stained for pFAK (Y397) (Y397) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Noting that cells showed the production of multiple projections when treated with acidic buffered media. Three different biological trials (n=3) were carried out. Images were taken using an ECHO revolved microscope at 60X objective. The scale bar is 30 µm.

3.8 The localization of phospho-paxillin (Y118) altered by acidity and acidity combined with lactate in HUVEC and B16F10 cell migration, attachment, and spreading.

Paxillin is a multifunctional adaptor protein associated with focal adhesions that undergoes phosphorylation at various tyrosine residues, predominantly facilitated by the actions of FAK and Src kinases. This phosphorylation process is essential for the recruitment of additional signaling molecules and structural proteins to focal adhesions. paxillin regulates the signals that govern the formation and breakdown of these focal adhesions, processes that are vital for cell migration, attachment, and spreading.[144] To investigate the alterations that occur on paxillin phosphorylation, a further step in this project was taken. Immunocytochemistry (ICC) was used to stain for the phospho-paxillin protein (pPAX (Y118)). The localization of pPAX (Y118) was investigated in migrated, attached, and spread cells in both HUVEC and B16F10. For both cell models, it was observed that the location of pPAX (Y118) was altered when migrated cells were treated with pH 6.4 buffered medium during the time course of wound assay experiment, pPAX (Y118) is mainly found at the outer boundary of the cell (cell periphery). This positioning is linked to the formation of pPAX (Y118) at the edges of the cell. The treatment of HUVEC cells with pH 6.4 and 40 mM lactate results in localization of pPAX (Y118) in central regions besides the cell periphery. This suggests alterations in pPAX (Y118) dynamics and actin cytoskeleton organization in response to the combined influence of acidity and lactate. The comparable impact on pPAX (Y118) localization seen when using 40 mM lactate in a pH 7.4 buffer, as opposed to acidic conditions, may be credited to lactate's ability to prompt metabolic and signaling alterations in the cells. These alterations alter the cellular reaction to acidic stress, leading to pPAX (Y118) activation and the rearrangement of focal adhesions and actin stress fibers **(Figure 3.19)** and **(Figure 3.20)**. An

additional step in the investigation included examining the alterations in pPAX (Y118) distribution as cell morphology (cell shape and elongation) changed in reaction to **(Figure 3.21)** and **(Figure 3.22)**. A distinct elongation of HUVEC and B16F10 cells in acidic environments and with lactate was observed when studying the effect of acidity on the cells when a single-cell migration assay was carried out to study the changes in cell morphology in both cell models. HUVECs exhibited more pronounced elongation in response to acidic treatment compared to normal treatment. Conversely, B16F10 melanoma cells display less noticeable elongation in acidic conditions alone, owing to their adaptation to such environments, but demonstrate increased elongation when lactate is added. Studying the pPAX (Y118) and actin fibers, actin stress fibers formed under acidic treatment in both cell models but with greater significance in HUVECs compared to B16F10. When it comes to the pPAX (Y118) location, under mildly acidic conditions (pH 6.4), pPAX (Y118) is primarily found at the periphery of the cells in both cell models, with a more significant presence in HUVEC cells, while pPAX (Y118) was in both cell periphery and cell body when cells were treated with pH 7.4 buffered media. This localization correlates with enhanced focal adhesion formation at the cell margins, facilitating the stabilization of cell attachment to the extracellular matrix (ECM) during stress conditions, such as low pH. A similar effect was noted when cells were exposed to an acidic buffer and 40 mM lactate, reducing aggregated pPAX (Y118). The same phenomenon was observed when cells were treated with 40mM lactate in a pH 7.4 buffer mimicking the same effect when cells were treated with acidic treatment **(Figure 3.23)**, **(Figure 3.24)**, **(Figure 3.25)** and **(Figure 3.26)**.

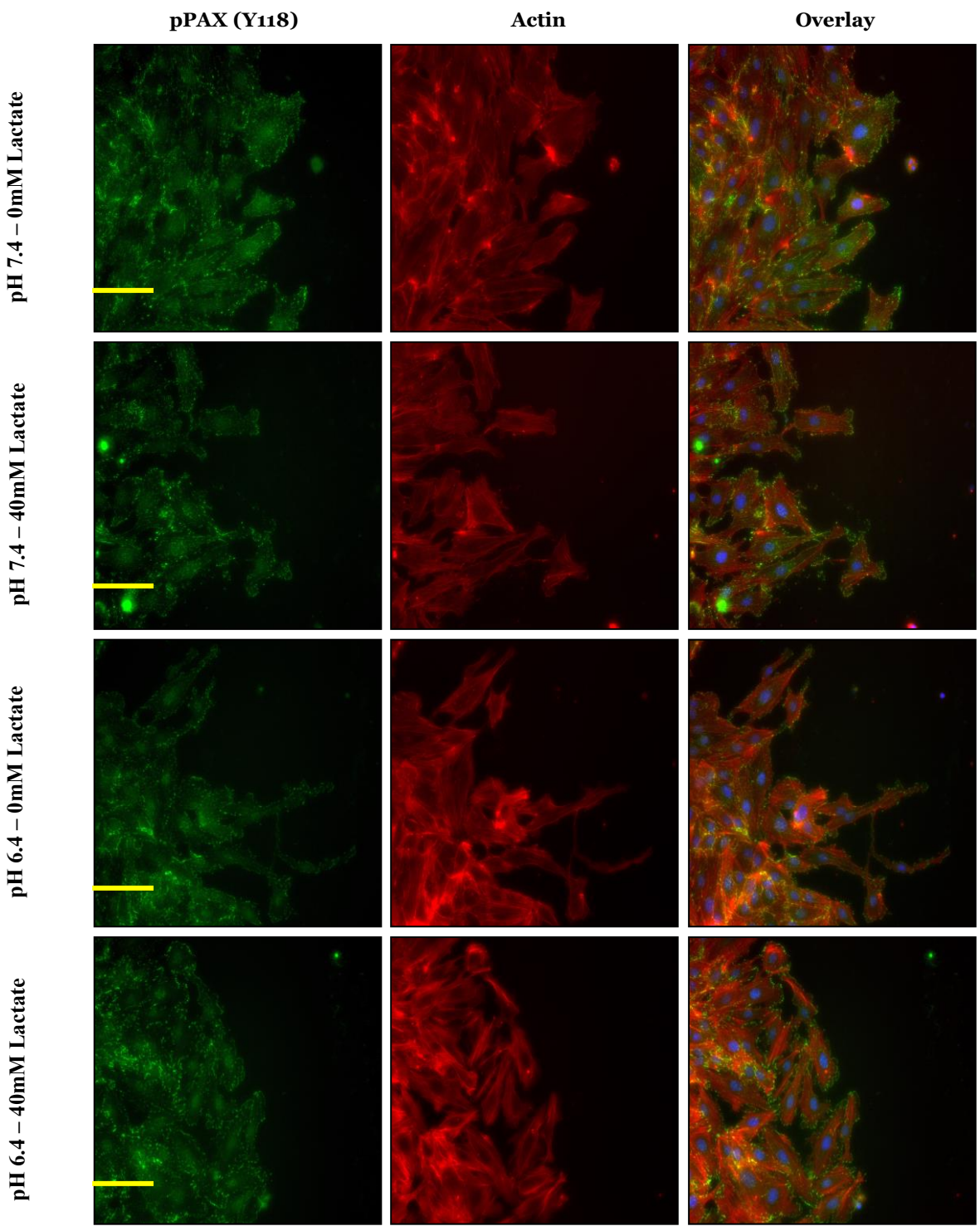

Figure 3.19: Representative images for **HUVEC** stained for pPAX (Y118) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Four different biological trials (n=4) were carried out. Images were taken using an ECHO revolved microscope at 20X lens objective and a 90µm scale.

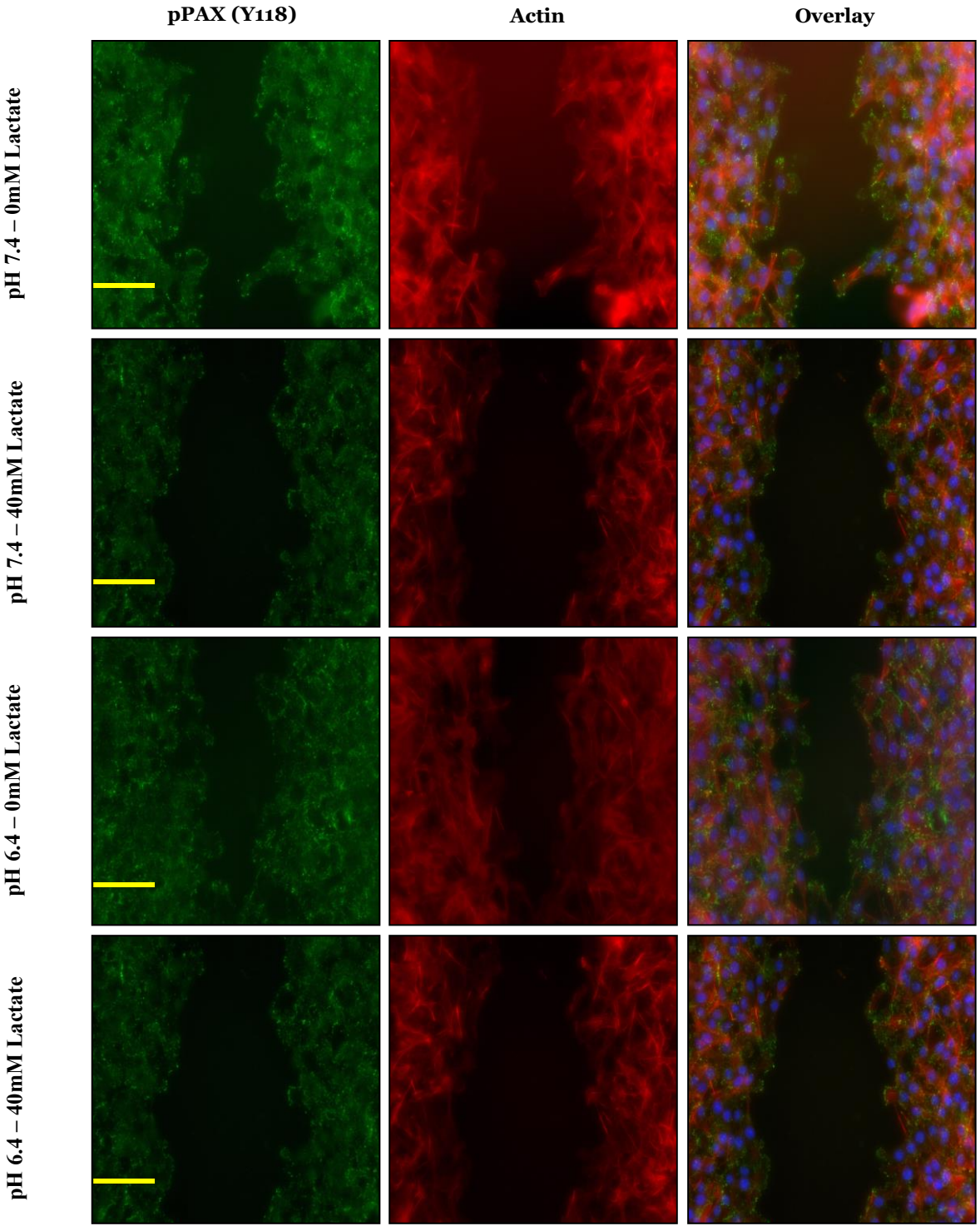

Figure 3.20: Representative images for **B16F10** stained for pPAX (Y118) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Four different biological trials (n=4) were carried out. Images were taken using an ECHO revolved microscope at 20X lens objective and a 90µm scale.

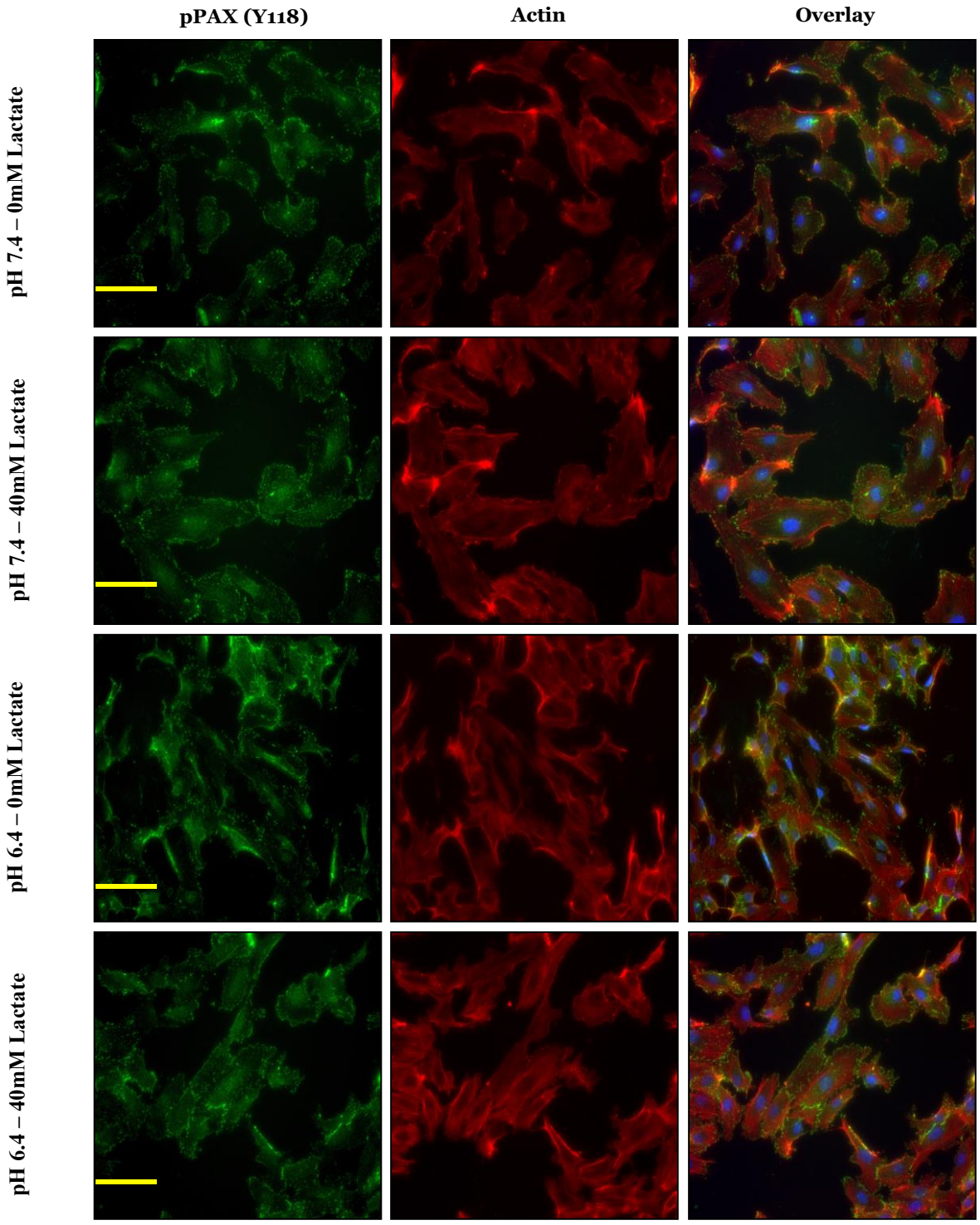

Figure 3.21: Representative images for **HUVEC** stained for pPAX (Y118) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC) to study morphology changes via single cell morphology assay. Four different biological trials (n=4) were carried out. Images were taken using an ECHO revolved microscope at 20X lens objective and a 90µm scale.

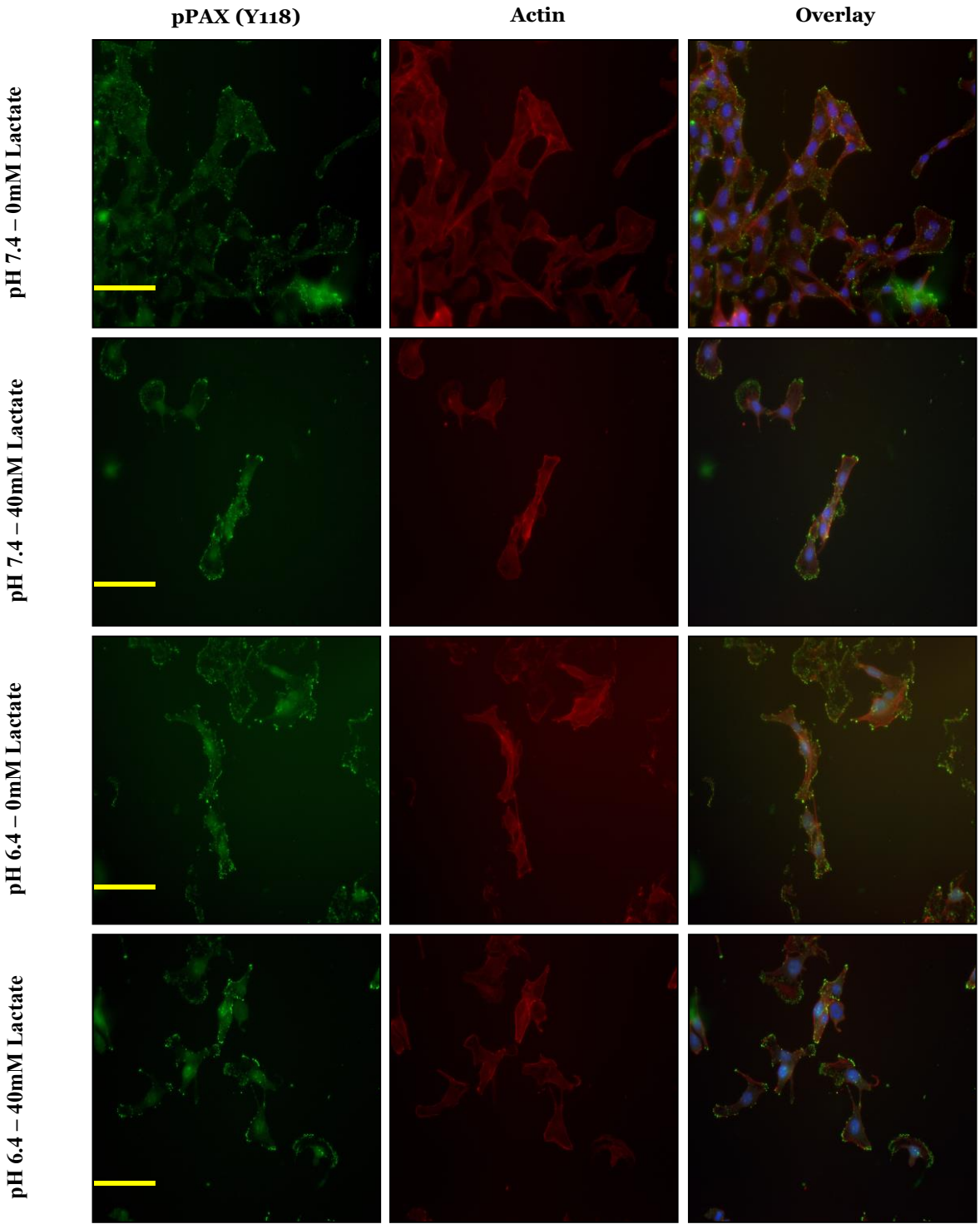

Figure 3.22: Representative images for **B16F10** stained for pPAX (Y118) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC) to study morphology changes via single cell morphology assay. Four different biological trials (n=4) were carried out. Images were taken using an ECHO revolved microscope at 20X lens objective and a 90µm scale.

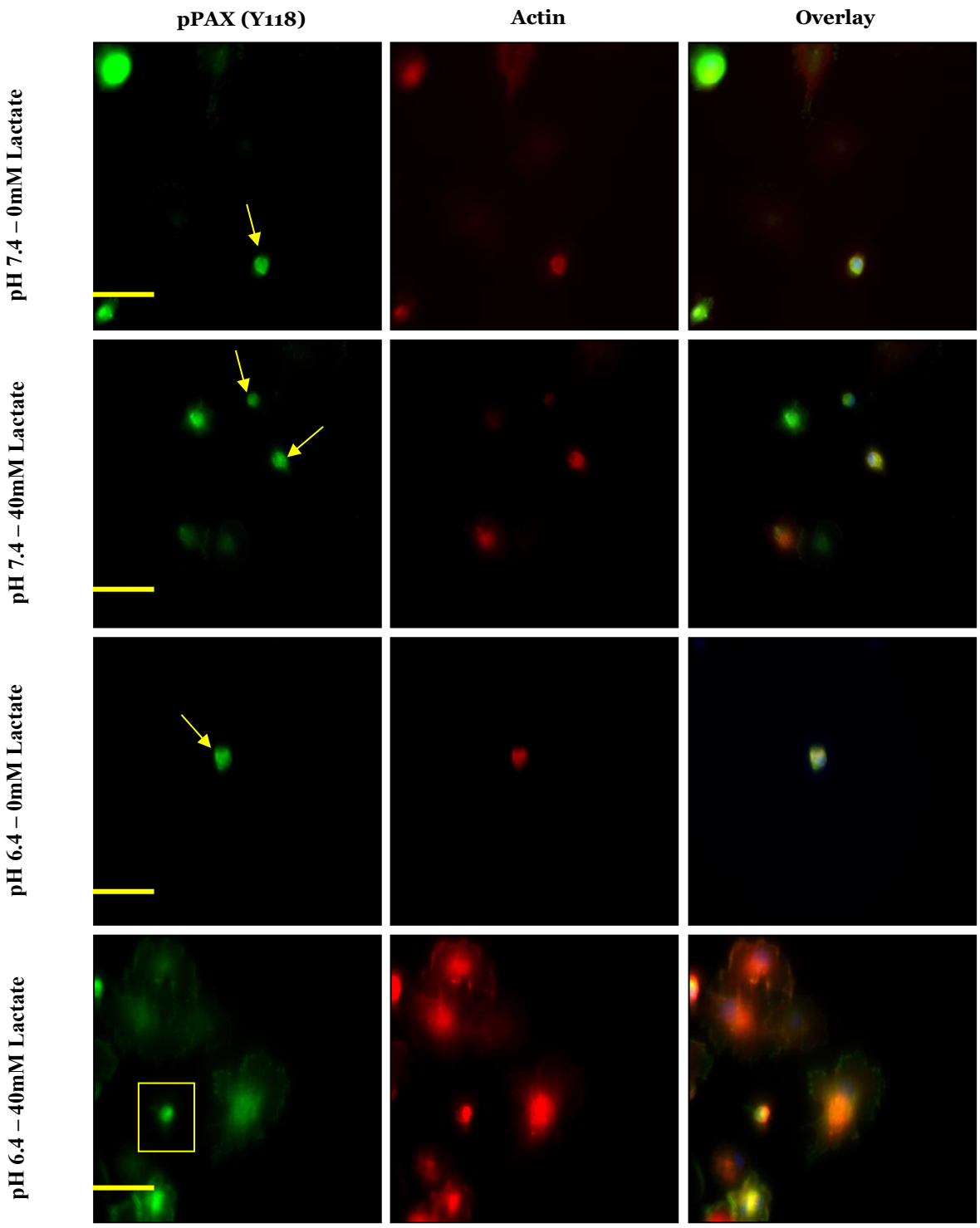

Figure 3.23: Representative images for ***round*** **HUVEC** via cell attachment assay. Cells stained for pPAX (Y118) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Four different biological trials (n=3) were carried out. Images were taken using an ECHO revolved microscope at 20X objective. The scale bar is 90 µm.

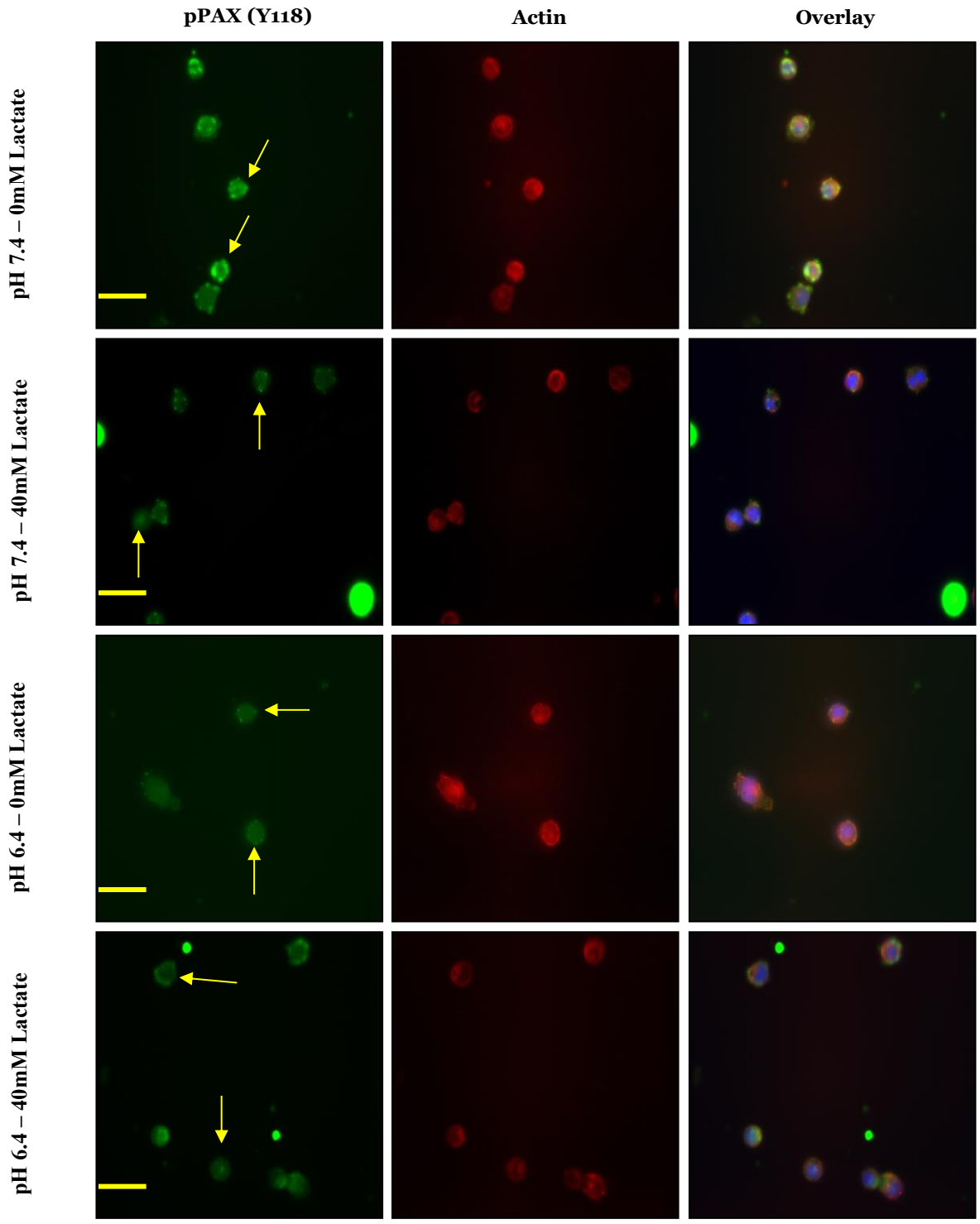

Figure 3.24: Representative images for ***round*** **B16F10** via cell attachment assay. Cells stained for pPAX (Y118) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Noting that cells showed the production of multiple projections when treated with acidic buffered media. Three different biological trials (n=3) were carried out. Images were taken using an ECHO revolved microscope at 40X objective. The scale bar is 50 µm.

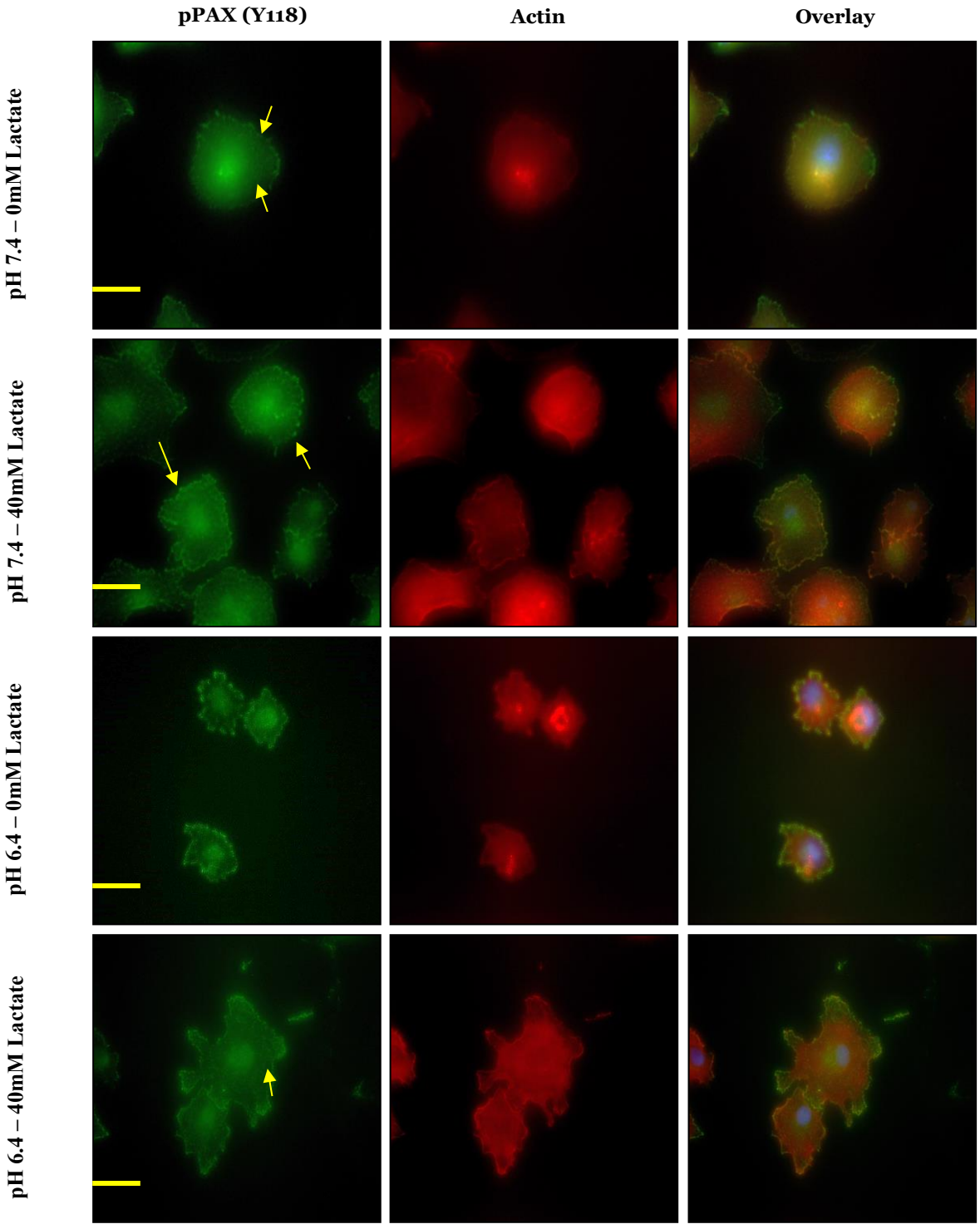

Figure 3.25 Representative images for ***Spread*** **HUVEC** via cell attachment assay. Cells stained for pPAX (Y118) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Noting that cells showed the production of multiple projections when treated with acidic buffered media. Three different biological trials (n=3) were carried out. Images were taken using an ECHO revolved microscope at 40X objective. The scale bar is 50 µm.

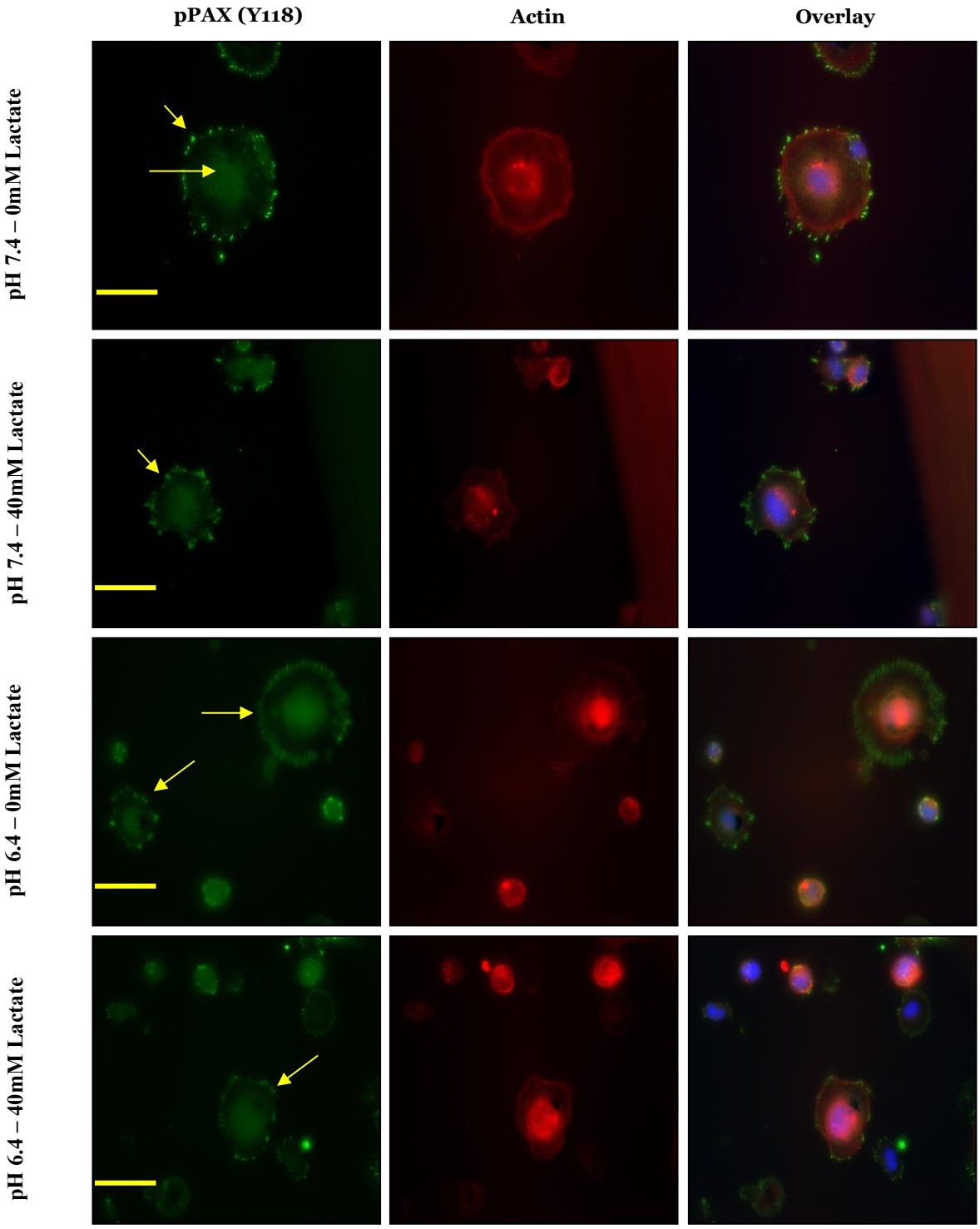

Figure 3.26 Representative images for ***Spread*** **B16F10** via cell attachment assay. Cells stained for pPAX (Y118) localization when cells treated with acidic buffer with/without Lactate by Immunocytochemistry (ICC). Noting that cells showed the production of multiple projections when treated with acidic buffered media. Three different biological trials (n=3) were carried out. Images were taken using an ECHO revolved microscope at 60X objective. The scale bar is 30 µm.

3.9 The phosphorylation level was reduced in FAK and PPAX when HUVEC and B16F10 cells were treated with acidic buffered media. The lactate effect combined with acidity caused a further reduction of pFAK (Y397) and pPAX (Y118) levels in B16F10 but not HUVEC.

The use of Western blot analysis to examine alterations in the expression and phosphorylation levels of proteins such as focal adhesion kinase (FAK) and paxillin (pPAX) in cells treated with various acidic buffers is an essential method. Acidic environments can change how cells communicate, which can have an impact on the localization, stability, and post-translational changes like phosphorylation of proteins.[146] Focal adhesion kinase (FAK) and paxillin (pPAX) phosphorylation levels and intracellular localization are important variables in controlling cell adhesion and spreading, especially in stressful situations like being treated with acidic buffers or acidic buffers with high lactate content. For that reason, an additional measure was taken to evaluate the effects of acidic treatments on the phosphorylation levels of paxillin and focal adhesion kinase in this project. In HUVECs, FAK and pPAX phosphorylation have been shown to decrease under acidic conditions, but phosphorylation enhancement was observed when lactate was added to the acidic buffer when cells after 1 hour attachment were tested **(Figure 3.27)**. In B16F10, FAK and pPAX phosphorylation have been shown to decrease under acidic conditions, and further decrease was observed when lactate was added to the acidic buffer when cells after 1 hour attachment were tested **(Figure 3.28)**, showing opposite effect for the combination of acidity and lactate on both cells model in the contest of this assay.

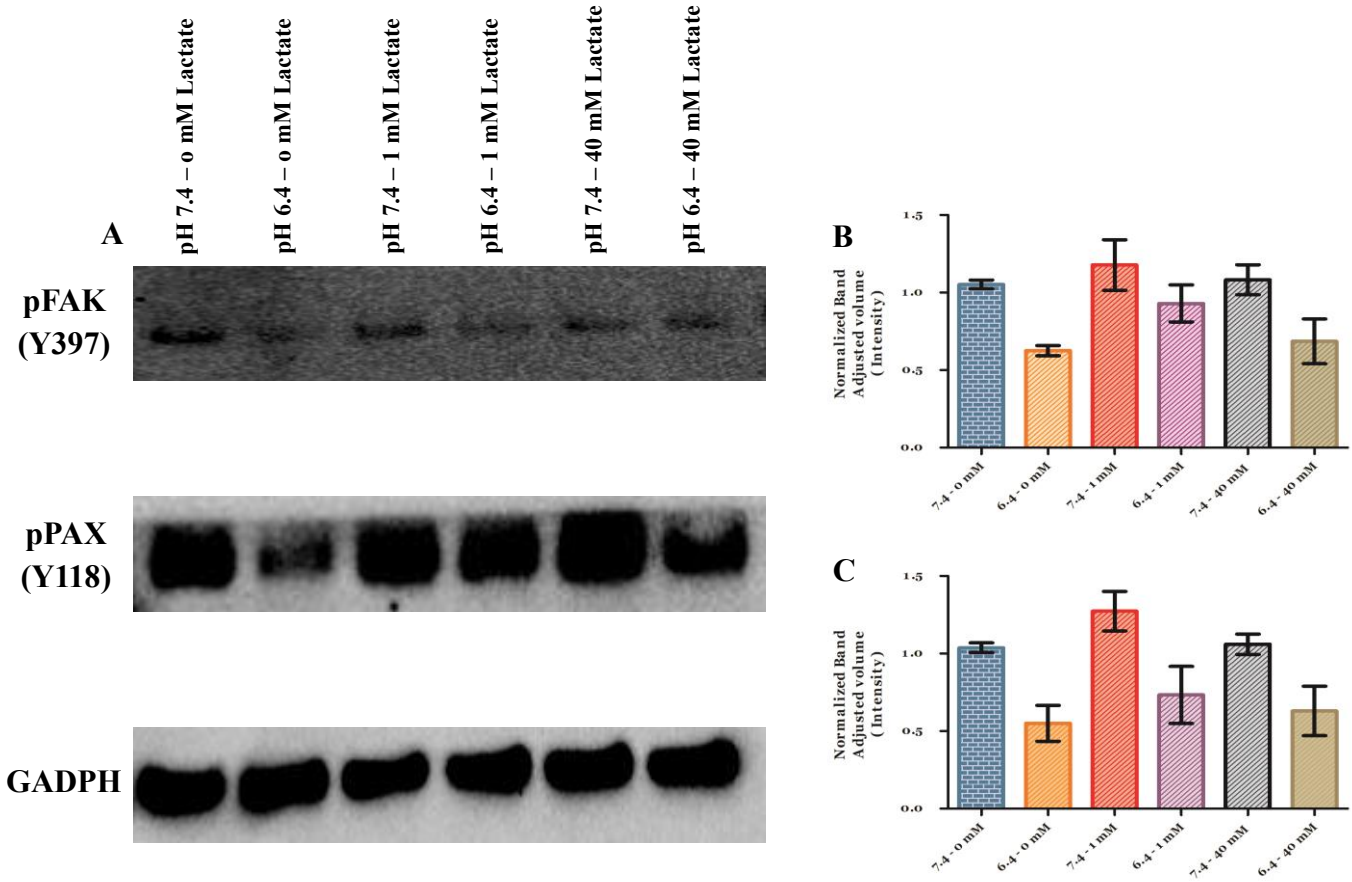

Figure 3.27: **(A)** Representative images for the changes in the phosphorylation levels of FAK and PPAX in ***HUVEC*** via 1 hour cell attachment assay. **(B)** Representative bar graph showing the changes of levels of pFAK (Y397) when cells treated with different acidic buffered media with and without lactate. **(C)** Representative bar graph showing the changes of levels of pPAX (Y118) when cells treated with different acidic buffered media with and without lactate. Four different biological trials (n=3). Western blot images were taken using Bio-Rad Chemi Doc Touch Image System Gel Imaging System. Images were analyzed using image lab 6.1 software.

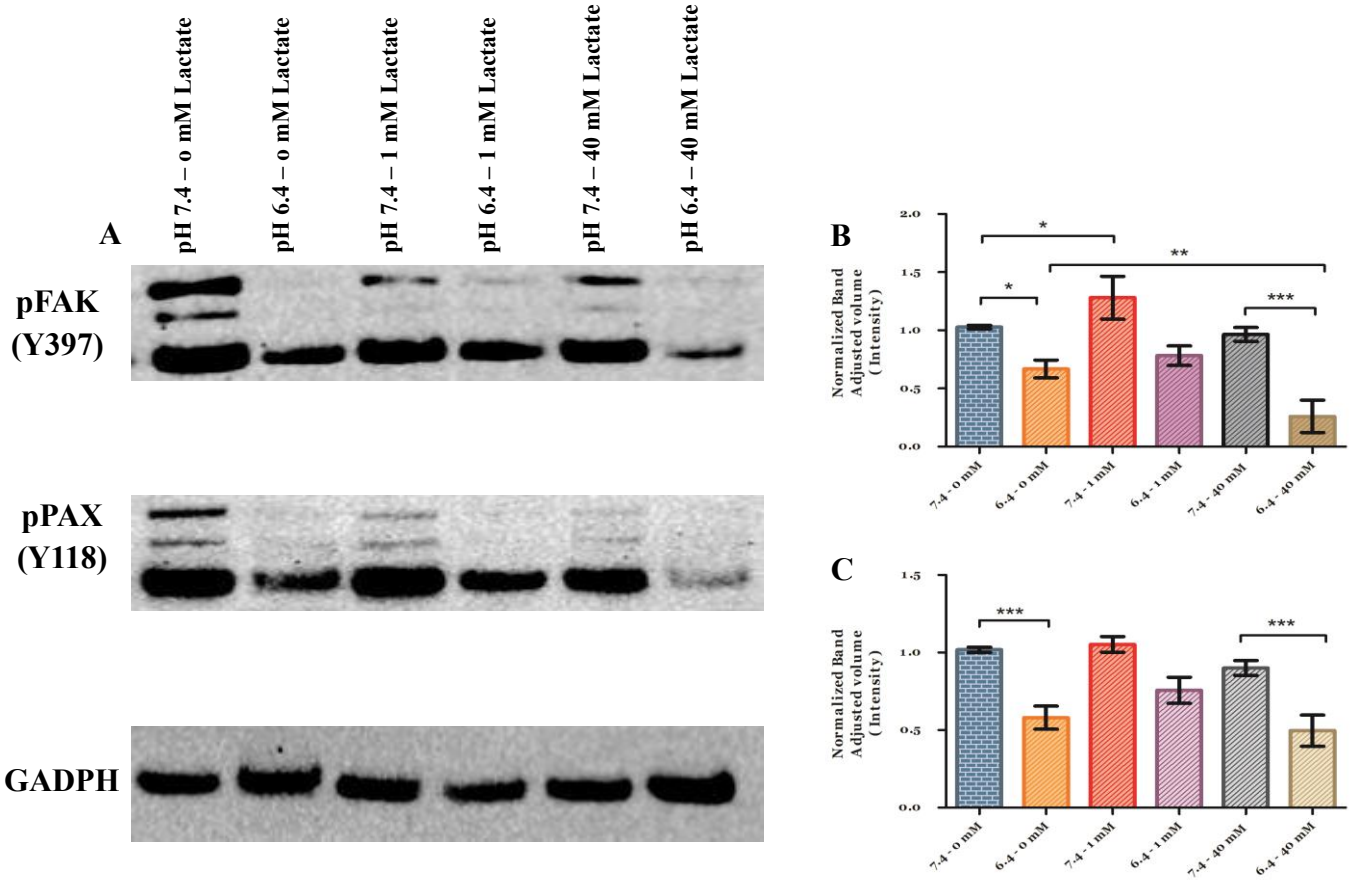

Figure 3.28: **(A)** Representative images for the changes in the phosphorylation levels of FAK and pPAX in ***B16F10*** via 1 hour cell attachment assay. **(B)** Representative bar graph showing the changes of levels of pFAK (Y397) when cells treated with different acidic buffered media with and without lactate. **(C)** Representative bar graph showing the changes of levels of pPAX (Y118) when cells treated with different acidic buffered media with and without lactate. Four different biological trials (n=3). Western blot images were taken using Bio-Rad Chemi Doc Touch Image System Gel Imaging System. Images were analyzed using image lab 6.1 software.

Chapter 4: Discussion

4.1 Introduction

The tumor microenvironment (TME) is known to have elevated proton and lactate levels due to the high rate of glycolysis even in oxygen-rich environments,[147] which has a major effect on vital cellular functions including migration, attachment, and spreading[148]. These functions play a significant role in normal cell angiogenesis and cancer metastasis. Previous studies in different cell types showed that acidic microenvironments either promote[149,150,151] or reduce[152] cell migration and attachment due to the modifications in the activation, expression, and distribution of important proteins such as FAK, pPAX, and actin.[153,154] It should be highlighted that there have been very few studies focused on the impact of lactate on cell migration,[155,156,157,158] and none specifically addressed the effect of lactate on the different migration modes of HUVEC and melanoma cells. Therefore, in this study, we investigated the effect of an in-vitro acidic microenvironment with or without high levels of lactate on the morphological changes including collective migration, directional migration, random motility three distinct modes of cell movement, cell attachment, and cell spreading, in addition to the mechanistic alterations involving the key proteins such as pFAK, pPAX, and F-actin in both HUVEC and B16F10 cell models. A better understanding of these cellular dynamics, functions, and protein translocation and post-translational modifications would provide guidelines for novel and improved anticancer targets. Novel anticancer treatment strategies targeting tumor cell migration, invasion, and angiogenesis would lead to a higher survival rate in cancer patients.

4.2 Cell Migration: Collective, Directional, and Random Motility

4.2.1 Collective and Directional Cell Migration

Qualitative and quantitative time-lapse imaging besides a quantitative wound closure analysis showed a suppression in the collective migration rate with acidity compared to the physiological pH in HUVECs and B16F10 cells. The slow migration is possibly attributed to the loss of usual dynamic cell movement, as a difficulty of tail detachment was observed in the migratory cells in the acidic microenvironment. Interestingly similar anti-migratory effect was observed in the presence of high levels of lactate under physiological pH, and the combined effect of acidity and high lactate level showed a more intense reduction in cell migration. Therefore, lactate itself was found to exert significant cellular stress and alter the physio-mechanics of the cells. Thus, high lactate levels produced by the metabolically active cancer cells in the TME are crucial in consideration for novel and improved anti-cancer therapies. These observations signify the intense cellular stress due to the inhibitory effect of acidity and lactate on cell migration in the TME. Collective migration in cancer was suggested to be the clinically prominent form of metastatic migration,[159] therefore we proposed that the effects of acidity and/or lactate on collective cell migration could lead to the development of therapeutic approaches that simultaneously inhibit migration, angiogenesis, and limiting metastasis.

Cell migration occurs in various modes depending on the cell type, migration context and the cell's microenvironment. Those migration modes include collective migration,[129] directional migration,[108] and random motility.[130] The changes in the microenvironment caused different effects on the migration modes due to the effect of these changes on the cellular molecular dynamics inside the migrating cells.[160] The directional cell migration under gradient acidic conditions have been reported earlier however in the context of prolonged constant acidic with or without lactate

condition has not been explored so far.[161] Therefore, we found that cells collectively and directionally migrate slower towards the wound gap under prolonged constant acidic conditions than physiological pH. This suppression is possibly related to the disruption effect of acidity on the adhesion dynamics; thus, cells struggle to establish the traction forces needed for regulated directional movement.[162] A similar effect was observed in both HUVEC and B16F10 when cells were migrating at pH 7.4 combined with high levels of lactate indicating a similar effect of acidity. The distance reduction effect due to acidity was intensified with the combined effect of lactate and acidity, emphasizing the suppression effect of lactate in this framework of the assay. We suggest that (for our experimental conditions and cell models) it could be that the elevated lactate levels disrupted the integrin-mediated adhesion to the extracellular matrix, altering the phosphorylation events at focal adhesion sites (pFAK (Y397) and pPAX (Y118)), which are critical for collective and directional migration. This alteration caused a decrease in migration efficiency.

4.2.2 Random Motility

As reported in previous studies, increased random motility assists in the spread of cells from primary tumors to secondary sites, [130,163] in addition to the role of cell motility in immune responses where irregular cell motility can lead to impaired immune responses, causing conditions like chronic inflammation or autoimmune diseases.[164] In this instance, we found that tumor microenvironment acidosis suppresses HUVEC random motility while having a reverse impact on B16F10 cells. This could be related to enhanced cancer cell motility in cancer cell progression and metastasis. In contrast, less motility and enhanced directionality in HUVECs are crucial for forming new blood vessels (angiogenesis) mainly during the initiation stage, where endothelial cells respond to chemical signals (like VEGF).[165] Interestingly, the addition of lactate to acidic microenvironment further decreases the random motility in HUVEC but increases it in B16F10.

This suggests that lactate might modulate or normalize cell behavior in specific cancer types, potentially influencing metastasis, in addition to improving more efficient angiogenesis by constraining random motility and improving HUVEC directionality as a result of a more efficient angiogenesis beginning stage. Thus, understanding the effect of acidosis and lactate enables more accurate approaches for design of more precise therapies targeting cell migration pathways specific to cancer metastasis, abnormal angiogenesis, and immune regulation.

4.3 Cell Morphology

4.3.1 Polarizing Index (Cell Elongation)

Previous data showed that excessive acidosis caused cellular structural and morphological changes.[166,167] Our data showed elongated cell morphology with a higher polarizing index for both HUVEC and B16F10 in the acidic microenvironment during cell migration. Moreover, this elongation was accompanied by difficulty in cell tail detachment in B16F10, and a more extreme effect showed in the tail separation from the cell body in HUVEC. This can be explained by the possible effect of acidity in disrupting the actin cytoskeleton dynamics altering cell polarity compared to normal pH. This assumption aligns with previous investigations about the effect of external cellular pH on the cytoskeleton rearrangement.[153] Moreover, acidosis works as a disruption effect that causes alterations in the traction forces dynamics responsible for regulating cell rear detachment, causing the difficulty in tail detachment, and therefore reduction in cell migration.[162] We recorded another new observation showing that high levels of lactate in a normal pH caused the same elongation effect in both cell models as acidosis but didn't have this effect when combined with acidity. The morphological changes due to lactate were mentioned in one study, which aligns with our data.[168] We therefore suggest that lactate has the same disruptive effect

as acidosis, causing cells to elongate, while acidity—rather than lactate—is the primary source of stress that results in these morphological changes in acidic environments.

4.3.2 Cell Protrusions

In addition to cell elongation, cell protrusions are vital in the process of cell migration dynamics. Based on the cellular context and the microenvironmental conditions, multiple cell protrusions could have different impacts on cell migration, multiple protrusions can enhance a cell's ability to navigate complex environments or block the efficiency of cell movement.[169] It was reported that disruption in cell protrusion caused dysregulation in the cell's polarity leading to the forming of multiple competing protrusions in the same cell, causing instantaneous interruption during migration.[140] This is mainly caused by altering the dynamics of the actin cytoskeleton (polymerization) and adhesion complexes.[140] The effect of acidity on the regulation of actin polymerization was well studied,[170,171] but the effect of lactate in actin polymerization remains unclear and not investigated. The data about the effect of lactate in this study will be a significant addition to the understanding of actin cytoskeleton dynamics and cell protrusions in the tumor microenvironment. We found that acidosis has a disruptive effect on the cellular protrusion dynamic. It was observed that the effect is disruptive in HUVEC rather than B16F10 cells. This Caused normal cells to form multiple protrusions (filopodia) in the front that forces the cell forward during migration. This shows that acidity is a source of altering the dynamic of actin fiber and adhesion formation. Also, this difference in the effect between HUVEC and B16F10 cells can be explained based on the adaptive capacity of melanoma cells in harsh environment . They can alter cell signaling pathways to make them more reliant on pH changes in the surrounding environment.[172] Interestingly, when coupled in acidic media, lactate reversed the acidity effect; but, in a normal pH environment, the protrusion dynamic remained unaffected. This suggests that

the effect of lactate on the actin protrusion dynamic is effective only when combined with acidity. Moreover, we suggest that the lactate accumulation in the acidic microenvironment alters specific cellular signaling pathways, including the ones related to actin polymerization and depolymerization, and adhesion formation such as PI3K/Akt signaling pathway.[173] Integrating all the earlier cell morphology data, we propose that the increase of cell elongation due to the acidity effect could serve to benefit tumor angiogenesis by increasing the cell elongation, which was reported to play a major role in the extension of the sprouting vein occurs during the sprouting phase of angiogenesis.[174] On the other hand, acidosis increases cell protrusions and could be one factor that reduces cell directional migration, in addition to the alterations in the actin cytoskeleton regulation, where cells swing between competing cellular protrusions resulting in cycles of elongation and retraction causing a slower directional migration.[175] The combination of lactate and acidity eases the effect of acidity on the protrusion formation but keeps the competing cellular protrusion formation higher compared to cells treated with physiological pH. Collectively, this disrupts the cells' capacity to uphold cohesive polarity and synchronized movement, resulting in enhancement in the cell elongation to serve the sprouting of new blood vessels at the expense of the enhancement in the collective and directional migration.

4.4 Cell Attachment and Spreading

Cell attachment and spreading are foundational stages of cell motility and migration. To migrate, cells first attach to the extracellular matrix or neighboring cells through focal adhesions, followed by spreading, where cell membrane extension and cytoskeleton rearrangement occur to stabilize the attachment sites and initiate a forward cell movement.[101,102] The delay in cell attachment and spreading was barely investigated, one study showed the effect of acidity in the suppression of the attachment and spreading of fibroblasts.[176,177] The effect of lactate on cell

attachment and spreading was not investigated. We found that cell attachment and spreading were delayed in both HUVEC and B16F10 cells. Lactate in a physiological pH microenvironment showed the effect of acidosis in HUVEC but not in B16F10, where there was no significant effect of lactate. Adding lactate to the acidic microenvironment caused a reduction in the effect of acidosis, by enhancing both attachment and spreading in HUVEC and B16F10 cells. These results suggest that acidosis possibly disrupts the normal interaction between the cell and its extracellular environment by altering the binding efficiency of integrins in the extracellular matrix and other adhesion molecules on the cell surface. Moreover, lactate works differently based on the pH of the microenvironment and cell type. This could be due to lactate interfering with pH-sensitive proteins involved in adhesion such as FAK and PPAX, or by countering pH stress through lactate-mediated metabolic pathways or by stabilizing cytoskeletal components. These results have important consequences for research on cancer and inflammatory diseases. In the context of cancer, focusing on acidity-sensitive proteins or lactate metabolism may hinder metastasis by stopping abnormal adhesion and migration. For inflammatory disorders, strategies could be implemented to restore cell-matrix interactions[178] that are affected by acidosis or to regulate the role of lactate in inflammation, which could help reduce chronic inflammation and tissue damage in conditions such as arthritis and fibrosis. The findings highlight one of the causes of the slower and less cohesive cell migration in acidosis microenvironments with high lactate levels.

4.5 Cell Signaling Proteins (FAK and Paxillin) and Cytoskeleton Alterations

4.5.1 Introduction

Cell migration, attachment and spreading depend on the complicated interactions between several key proteins that control cell signaling and the cytoskeleton. These include actin, pFAK (Y397), and pPAX (Y118). Actin is the primary component of the cell's cytoskeletal structure; it

is the base of the mechanical forces required for cell movement and shape changes. Cell protrusions are driven by actin polymerization at the leading edge of the cell, which promotes migration and spreading.[179] pFAK (Y397) and pPAX (Y118) are the phosphorylated and activated forms of FAK and paxillin. Tyrosine 397 (Y397) is the primary autophosphorylation site in FAK, and Tyrosine 118 (Y118) is the primary phosphorylation site in paxillin, the phosphorylation of those sites generates a high-affinity binding site for other signaling proteins that regulate pathways like the PI3K/Akt cascade, which regulates cell survival, proliferation, and motility.[180,181,182] pFAK (Y397) and pPAX (Y118) are crucial components of Integrins mediating between cells and ECM. pFAK (Y397) is a key regulator that initiates signaling cascades, reinforcing the assembly of focal adhesions and actin cytoskeletal remodeling. Simultaneously, pPAX (Y118) acts as a scaffold protein that stabilizes these adhesion sites and regulates actin dynamics, thus regulating cell attachment, spreading, and migration.[143,183,184] Together, these proteins coordinate the complex series of events that allow cells to adhere to their surroundings and a forward movement. Understanding the effect of acidity with or without lactate on actin, pFAK (Y397), and pPAX (Y118) is vital in exploring cell behaviors under physiological and pathological conditions, such as wound healing, cancer metastasis, and inflammation.

4.5.2 Actin Dynamic Alterations

While the effect of acidosis upon those major proteins during migration was discussed previously for different types of cells, the effect of acidosis with and / or without lactate on those proteins was not investigated. It was reported that acidity can trigger signaling pathways, such as through the GPR4 receptor, to regulate cytoskeleton changes and cell migration [176,177]. We found that acidity significantly altered actin dynamics and its spatial distribution during migration and spreading in both HUVEC and B16F10. Under acidosis, Actin stress fiber formation was clear and prominent

in migrating cells, and at the periphery of the spreading cells. This suggests that acidosis has a reorganizing effect on the cytoskeleton of the cells. Moreover, it indicates that acidosis might serve as a cellular signal that triggers the reinforcement and enhancement of actin structures and actin polymerization, this finding aligns with previous data for both different cell lines and experiment circumstances.[185,186] Relating this finding to cell migration, the formation of actin stress fibers under acidosis causes an increase in cell contractility and rigidity.[187] According to a study, stress fiber-mediated cell stiffening may promote tumor growth in premalignant phases. Therefore, further research into the mechanical characteristics of tumor cells may open new research directions for the development of cancer-targeting treatments.[188] In a physiological pH microenvironment, lactate exhibited the same effects as acidosis in HUVEC, but not in B16F10, where high concentrations of lactate induced the formation of actin stress fibers. Interestingly, the combination of lactate with an acidic environment did not induce significant changes in actin stress fibers of migrating cells when compared to cells exposed solely to acidity. However, different effects for the combination of lactate and acidity were observed in spreading cells. In HUVEC cells, the combination of lactate with acidity led to the formation of actin aggregates within the region of the cell body, while also being distributed at the cell periphery in the form of fibers. On the contrary, in B16F10 cells, actin displayed a more uniform distribution throughout the cell body, resembling the organization seen under physiological pH conditions. These significant findings suggest that lactate influences cell behavior differently based on the cell type and the acidity of the microenvironment. Different cells have different metabolic signaling responses,[189] thus the different responses suggest that lactate modulates actin dynamics during cell spreading in a cell type-dependent manner under acidic conditions. Endothelial cells are highly responsive to their microenvironment. When exposed to high levels of lactate at physiological pH, HUVECs may

experience metabolic changes and shifts in signaling pathways, such as the activation of lactate receptors (like GPR81). These changes possibly trigger signaling cascades leading to cellular contractility and actin dynamics, resulting in acidosis-like effects even at physiological pH.

4.5.3 FAK and paxillin Distribution Alterations

Phosphorylated FAK (pFAK (Y397)) and phosphorylated paxillin (pPAX (Y118)) are key regulators of cell migration and attachment, linked to actin cytoskeletal dynamics. The focal adhesion signaling initiated when pFAK (Y397) recruit proteins like paxillin, followed by the phosphorylation of paxillin to modulate the interaction with actin-regulatory molecules.[143,190,191] This phosphorylation cascade enables cells to extend protrusions and generate traction forces essential for attachment and movement[183,184]. In a very interesting study, it was found that pFAK (Y397) and pPAX (Y118) form separate subregions within the focal adhesions and influence cell adhesion and migration. Moreover, the phosphorylation states of paxillin and FAK affect their specific distribution and dynamics, which play a critical role in the assembly and stability of focal adhesions, ultimately impacting cellular motility[190,191]. based on those findings, we investigated the effect of acidosis and lactate on the pFAK (Y397) and pPAX (Y118) distribution and phosphorylation states of FAK and PPAX. We found that acidity altered both pFAK (Y397) and pPAX (Y118) locations during cell migration, attachment and spreading. Cells migrating and spreading under acidosis showed less pFAK (Y397) and pPAX (Y118) sites, more prominent on the cell peripheral compared to cells in normal pH microenvironment, where pFAK (Y397) and pPAX (Y118) sites were located in both cell body and cell peripheral. Moreover, acidosis caused larger pFAK (Y397) and pPAX (Y118) structures in certain spots on the cell peripheral. This suggests that acidosis impacts the phosphorylation of the FAK and pPAX and thus impacts the organization and mechanical properties of the actin cytoskeleton that affects cell migration and

spreading. The phosphorylation of FAK and paxillin is regulated by Rho family GTPases (a group of small signaling G proteins), which control the formation and turnover of focal adhesions and stress fibers[192]. We suggest that acidosis disrupts this regulation which may lead to the shift of the focal adhesion locations in the cells under acidic conditions. We suggest that the extreme cell elongation and tail separation from the migrating cell body, especially in HUVEC, that was observed due to acidosis is mainly driven by a slowdown in pFAK (Y397) turnover, which disrupts focal adhesion dynamics in the tail tip. This may have led to the prolonged stability of pFAK at the cell's trailing edge, preventing efficient detachment. In addition to that, the accumulation of actin stress fibers at the tip of the cell's tail, further anchors the cell in its elongated shape. We suggest that the interaction change due to acidosis between these factors disrupts the normal cell contractility and migration, leading to a distinct elongation phenotype as cells struggle to balance adhesion and cytoskeletal tension under acidic stress.

Lactate in a physiological pH microenvironment showed the effect of acidosis in more prominently in HUVEC rather than B16F10 cells, Lactate in a physiological pH microenvironment showed similar effects of acidosis in HUVEC, Cells migrating and spreading under acidosis showed less pFAK (Y397) and pPAX (Y118) sites, more prominent on the cell peripheral compared to cells in normal pH microenvironment, where pFAK (Y397) and pPAX (Y118) sites were located in both cell body and cell peripheral. The combination of lactate with an acidic environment led to increased phosphorylation of FAK and paxillin at multiple sites in the cell center, in addition to the periphery, compared to cells that were only exposed to an acidic environment. This suggests that lactate influences the phosphorylation of FAK and pPAX differently based on the cell type, cell status, and the acidity of the microenvironment.

4.5.4 FAK and paxillin Level of Phosphorylation

The distribution and localization of focal adhesions (FAK and PPAX), beside their level of phosphorylation at key sites such as FAK (Y397) and paxillin (Y118) is crucial for cell adhesion, spreading, and migration. Proper spatial distribution of the activated proteins ensures effective and coordinated cell migration, while well-controlled phosphorylation levels activate signaling pathways essential for adhesion turnover and force dynamic, which are crucial for cell attachment and directional cell movement.[193,194] Few studies showed that pH may directly regulate the phosphorylation levels of FAK, thereby influencing cell motility.[195,196] But the effect of lactate on the phosphorylation level of FAK and paxillin was not investigated. We found that the level of phosphorylation of FAK and paxillin in both HUVEC and B16F10 decreased. Suggesting that acidity has a blocking effect over the autophosphorylation of FAK, either due to the acidosis disruption effect upon cell-ECM interaction, or the alteration effect upon the mechanical forces' dynamics in the cell. These changes could alter the conformation of FAK, making the autophosphorylation sites in FAK less accessible, leading to a reduction in the phosphorylation of FAK. FAK functions as a kinase for other key hub and scaffolding proteins like paxillin. When FAK is phosphorylated, it subsequently triggers the phosphorylation of paxillin within focal adhesions[190,191]. Thus, the decrease of phosphorylation in FAK due to acidity caused a reduction of phosphorylation in paxillin as well. Interestingly, elevated lactate levels did not impact the phosphorylation of FAK or PPAX in HUVEC or B16F10 cells when present in a physiological pH environment. However, when combined with acidosis, lactate exhibits opposing effects: it reduces phosphorylation levels in B16F10 cells while elevating it in HUVECs. This suggests that the lactate effect is highly context-dependent, specifically influenced by the extracellular pH environment. Moreover, under normal pH conditions, lactate alone is not a significant regulator of

focal adhesion signaling pathways involving FAK and paxillin phosphorylation in these cell types. in HUVECs, the combination of lactate and acidosis caused an elevation in the phosphorylation levels of FAK and PPAX. This could suggest that lactate acts as a stimulus to enhance the activation of the focal adhesion complex in an acidic microenvironment.

Conclusion and Future Perspectives

In this research, the HUVEC and B16F10 cells were selected because of their distinct physiological and cellular significance in cancer studies. HUVECs are used as a model for endothelial cells, because of their primary role in angiogenesis. B16F10 melanoma cells are used as a transformed-cell model, as they are a well-established framework for examining the metastatic behavior of cancer cells. Data from both models yielded a comprehensive understanding and updated perspectives on how acidic conditions and high lactate levels in the tumor microenvironment (TME) affect essential cellular functions such as migration, attachment, and cell spreading.

We observed that the acidic microenvironment and elevated lactate levels led to significant morphological, migratory and mechanistic changes in both HUVEC and B16F10 cells. Acidosis caused cell elongation due to disrupted tail detachment during migration, particularly in HUVECs. Moreover, acidity led to disruption in the dynamic of protrusion formation especially in HUVECs, causing a compromise in the cohesive polarity and reducing collective and directional migration. Interestingly, the addition of lactate to acidic conditions further reduced these migration dynamics in both cell models, while it reversed the increased random motility seen in B16F10 cells under acidosis. These findings emphasize that lactate and acidity jointly alter cell morphology and migration behavior, highlighting their critical role in influencing tumor progression and angiogenesis. Given that cell motility and migration are crucial to angiogenesis, and considering the data gathered in this project, it is extremely important to explore the effects of lactate under varying pH conditions on angiogenesis including VEGFR signaling pathway, and the expression of angiogenic integrins like αvβ3,[197] as this has not been sufficiently investigated. This research area represents a novel perspective in the field of cancer research and normal cellular activity.

On the molecular level, our study highlighted that acidosis, and lactate distinctly affects the regulation of key proteins involved in cell attachment, migration, and spreading, particularly pFAK, pPAX and actin. Acidity promoted actin stress fiber formation, which increased cell rigidity and contractility, potentially linking this to cancer progression. Interestingly, lactate's influence differed based on cell type and acidity. These results suggest that lactate regulates actin behavior in a cell-specific and pH-dependent manners, highlighting the need for further research on cell-specific metabolic responses to acidosis and lactate during different cancer development stages. Exploring how acidosis and lactate impact actin-regulating proteins such as cofilin, Rac1, and RhoA will provide solid new data that focuses on the signaling pathways regulating the actin formation dynamic in the cell such as the Rho/ROCK. This may clarify how lactate regulates actin polymerization and metastatic behavior. This may also provide molecular targets for limiting metastasis by disrupting specific metabolic and cytoskeletal pathways in cancer cells.

When it comes to pFAK (Y397) and pPAX (Y118), Acidosis caused a disruption in the focal adhesion sites, causing them to shift mainly to the cell periphery, along with larger structures at these sites. Moreover, acidosis has inhibition effect upon the autophosphorylation of those proteins, likely due to disruptions in cell-ECM interactions or alterations in cellular mechanical dynamics. Interestingly, lactate differentially influenced the autophosphorylation and the location of the pFAK and pPAX in a cell-type-specific and acidity manners. lactate enhanced phosphorylation in HUVECs but reduced it in B16F10 cells indicating a complex regulatory role of lactate dependent on pH. These findings suggest that the microenvironment's pH and lactate levels could play critical roles in modulating focal adhesion dynamics, cell contractility, and migration, particularly under pathological conditions such as tumor progression. Future studies might examine the function of focal adhesion kinase (FAK) and paxillin in mechano-transduction,

using methods like atomic force microscopy (AFM) to measure cellular stiffness in microenvironment with high acidity and lactate. Furthermore, investigating pathways such as the FAK/Src and integrin-mediated signaling routes could improve the understanding about how focal adhesions affect cell contractility and movement in reaction to the tumor microenvironment.

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