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REGENERATIVE MEDICINE

Angiopellosis as an Alternative Mechanism of Cell Extravasation

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ABSTRACT

Stem cells possess the ability to home in and travel to damaged tissue when injected intravenously. For the cells to exert their therapeutic effect, they must cross the blood vessel wall and enter the surrounding tissues. The mechanism of extravasation injected stem cells employ for exit has yet to be characterized. Using intravital microscopy and a transgenic zebrafish line *Tg(fli1a:egpf)* with GFP-expressing vasculature, we documented the detailed extravasation processes in vivo for injected stem cells in comparison to white blood cells (WBCs). While WBCs left the blood vessels by the standard diapedesis process, injected cardiac and mesenchymal stem cells underwent a distinct method of extravasation that was markedly different from diapedesis. Here, the vascular wall undergoes an extensive remodeling to allow the cell to exit the lumen, while the injected cell remains distinctively passive in activity. We termed this process *Angio-pello-sis,* which represents an alternative mechanism of cell extravasation to the prevailing theory of diapedesis. STEM CELLS *2017;35:170–180*

SIGNIFICANCE STATEMENT

For a type of promising stem cell therapy, cells are injected directly into the blood stream. These stem cells cross the blood vessel wall into surrounding damaged tissue, where they are able to exert regenerative effects. The mechanism of how these injected cells escape the blood vessel remains unknown. This research paper provides the previously unknown answer to the question of what mechanism do therapeutic stem cells use to leave blood vessels when injected directly into the blood stream.

INTRODUCTION

As a promising strategy of regenerative medicine, stem cells are injected intravenously directly into the blood flow with the intended goal of the cells transmigrating across the blood vessel wall into the target tissue for repair [1–3]. Although such transmigration of injected cells through the blood vessel does occur, the exact mechanism is poorly understood [3, 4]. It has been postulated that the injected cells undergo a process similar to leukocytes/white blood cells (WBCs), termed diapedesis [5, 6]. In this process, WBCs inside the lumen squeeze through the endothelial barrier of the blood vessel into the surrounding tissue [7]. However, there are several distinct differences between the extravasation potential of intravenously injected cells and native white blood cells. WBC diapedesis extravasation is a relatively fast process occurring within minutes of an immune response, whereas injected foreign cells have been reported to take hours to days to extravasate from inside the blood vessel lumen [4, 6]. Another difference lies in the WBCs innate ability to extravasate as part of an inflammation/immune system response, in contrast to the lack of innate extravasation need of cells that are foreign to the blood stream.

Here we report a novel mechanism that non-leukocytic cells employ to extravasate when injected into the blood stream, which offers an alternative method complementing the prevailing diapedesis mechanism of extravasation. We have termed this extravasation process *Angiopellosis* (angio: relating to blood vessels; pello: push, drive out). This multi-step process (Fig. 1A) first involves the injected cells attaching to the endothelial lining of the vasculature after which the membrane projections of endothelial cells actively cover the foreign cells, prompting the expulsion of the injected cells into the surrounding tissue (Supporting Information Movie S1). Angiopellosis differs distinctly from diapedesis

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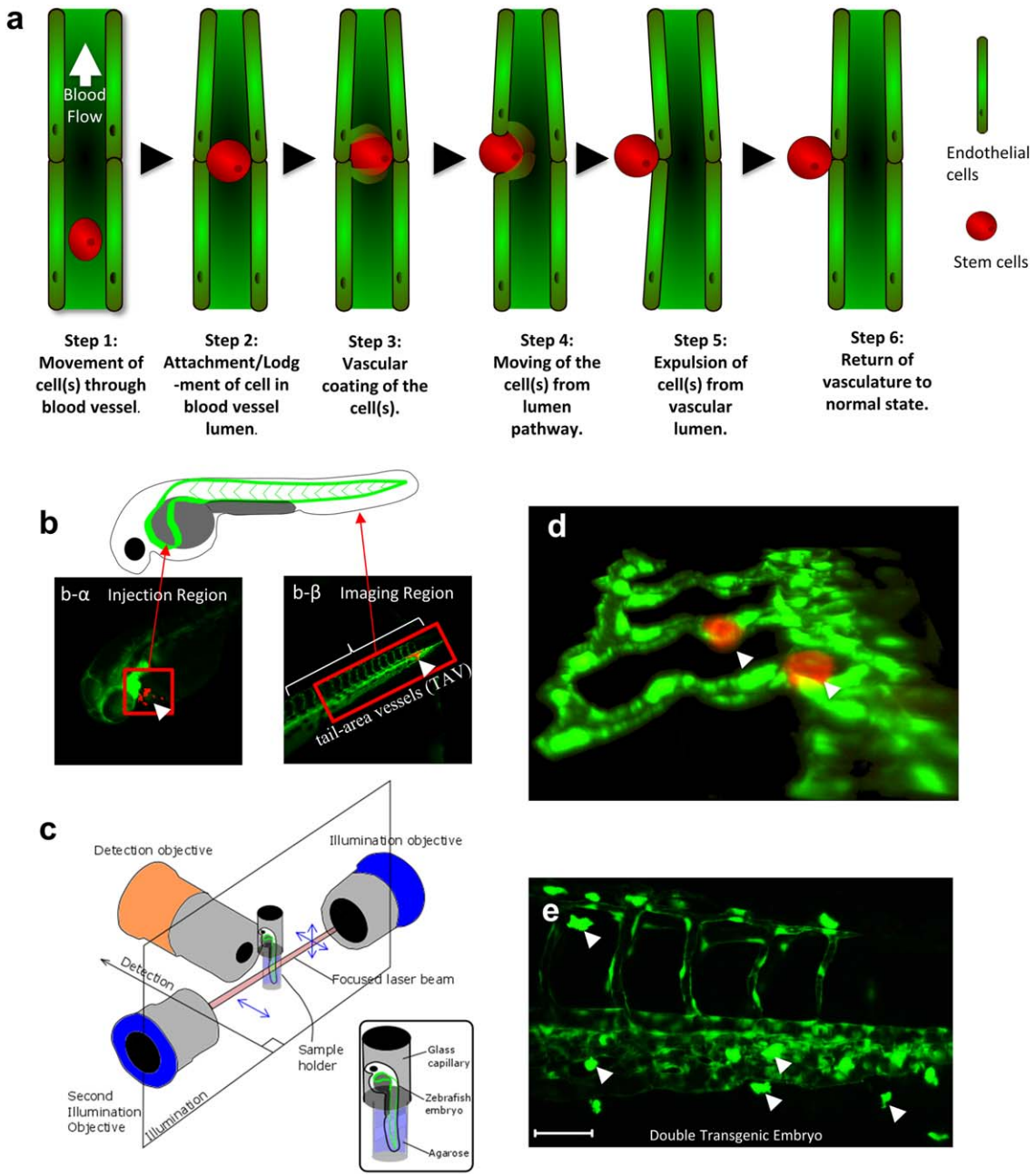

Figure 1. Intravital imaging of zebrafish vasculature using light-sheet microscopy for visualization of cell extravasation. **(A)**: Schematic illustration of angiopellosis in which endothelial cells extend protrusions around injected stem cells and actively expulse injected cell(s) into the parenchyma. Green cells represent green fluorescent protein (GFP)-expressing endothelial cells of the transgenic *Tg(fli1a:EGFP)* zebrafish embryo blood vessels; red cell represents injected stem cells; white arrow indicated blood flow. **(B)**: Schematic illustration of cell injection in *Tg(fli1a:EGFP)* zebrafish embryos. **(B-α)** Region of injection (red box) and injected cells (arrow) in the cardiac region following the duct of cuvier of a 48 hpf zebrafish embryo; **(B-β)** Region of imaging in the tail-area vessels (TAVs) zone (red box). **(C)**: Schematic showing the set-up of light-sheet microscopy. The living zebrafish is embedded in 1.3% agarose gel and positioned in front of the water-dipping detection lens. The sheet of light is generated by fast vertical scanning of a focused laser beam, and it illuminates a 4-μm-thick volume section of the fish. Fluorescence is recorded orthogonally to the light sheet with a wide-field detection arm equipped with a fast scientific complementary metal-oxide semiconductor camera. Fast volumetric imaging is performed by step-wise axial movement of the detection objective in synchrony with displacement of the light sheet while the specimen is kept stationary. **(D)**: Three-dimensional rendering of z-stack images of the tail-area vessels (green) with injected cells (red). **(E)**: Tail-area vessel (TAV) region of a double transgenic zebrafish *Tg(fli1a:EGFP)/(mpeg1:EGFP)* embryo, in which GFP is expressed simultaneously in vasculature and macrophages. Arrows indicate macrophages expressing GFP. Scale bar = 50 μm.

(Supporting Information Movie S2) in that the extravasation process is being carried out mainly by the activity of the blood vessel endothelial cells, rather than the circulating cells within the lumen as with white blood cells [8]. Angiopellosis also contains a key temporal difference from leukocyte diapedeses, taking hours rather than minutes to complete.

To elucidate the mechanism of cell extravasation we used a transgenic zebrafish line (Fig. 1B), *Tg(fli1a:EGFP)*, in which green fluorescence protein (GFP) is expressed exclusively in blood vessels [9]. Multiple cell types, labeled with the red fluorescent dye DiI, were injected in the blood stream of the transgenic fish embryo (Fig. 1B-α). The blood vessels with injected cells (Fig. 1B-β) were then imaged using lightsheet microscopy with 3D rendering capability (Fig. 1C). The lightsheet microscope allows for high resolution z-stack images to be taken while the embryo is submerged in a chamber for long (>16 hours) periods of time [10]. These images can be projected in three-dimensions to observe the exact location of cells in respect to blood vessels (Fig. 1D). Typical diapedesis occurred in injected or endogenous leukocytes (Fig. 1E), while angiopellosis occurred in injected cardiac stem cells (CSCs) from human, canine, and rat origins. Angiopellosis was also observed in polymer microspheres coated with cardiac stem cell membrane but not in non-coated microspheres.

To explore the molecular distinction between angiogenesis and diapedesis, we designed an experiment to inhibit a molecule that is required for diapedesis. CD11α is a 160-170 kD glycoprotein also known as αL integrin, or LFA-1 α chain. It is a member of the integrin family and forms a heterodimer with β2 integrin (CD18) [11]. CD11α is broadly expressed on WBCs as well as on mesenchymal stem cells (MSC) and cardiac cells and mediates intercellular adhesion and cellular activation [12, 13]. CD54 (ICAM-1), CD102 (ICAM-2), and CD50 (ICAM-3) are the ligands [11]. The antibody recognizes both activated and unactivated LFA-1 and inhibits the binding of LFA-1 to ICAM-1. The anti-CD11α antibody has been shown to inhibit the mixed lymphocyte reaction, leukocyte infiltration, and graft rejection [11]. When integrin CD11α was inhibited using an anti-CD11α antibody, the cardiac stem cells (CSCs) and MSCs retained the ability to extravasate through angiopellosis, while WBCs lost the ability to undergo diapedesis. This suggests that although cell membrane recognition is needed for angiopellosis, the molecular signal differs distinctly from that of diapedesis.

Results

Injected Cardiac and Mesenchymal Stem Cells Undergo Extravasation by Angiopellosis

Although there are prevailing beliefs that intravenously delivered cells leave the blood stream through diapedesis, as white blood cells do, evidence is lacking [4]. To uncover the extravasation mechanism we intravenously injected *Tg(fli1a:EGFP)* zebrafish embryos at 48 hours post fertilization (hpf) with DiI-labeled cardiac stem cells (CSCs) and mesenchymal stem cells (MSCs) isolated from human (Fig. 2C), canine (Fig. 2B), and rat (Fig. 2A) tissue. After cell infusion, zebrafish embryos were screened for labeled cells that had entered the tail-area vessels (TAVs) (Fig. 1B-β), which has been previously reported to be a desirable location for vascular imaging in embryos [9]. CSCs and MSCs were observed to extravasate (Supporting Information Movies S3, S4, S19) with a multistep process: first, they became lodged in the vessels, then the adjacent endothelial cells of the blood vessel extended protrusions (Fig. 2A, 2B, white arrowheads) to surround the cell and form an endothelial pocket. Subsequently, the endothelial protrusions actively remove the cell from the lumen into the surrounding parenchyma cavities (Fig. 2A, 2B). This active vascular expulsion process eventually led to cell extravasation. We termed this process *angiopellosis*. To reveal the distinctions between angiopellosis and the conventional diapedesis, we isolated viable white blood cells from rat whole blood, labeled, and injected them into the blood stream of the fish embryos [7]. In contrast to CSCs and MSCs, white blood cells underwent typical diapedesis (Fig. 2E): first attaching to the vessel wall, and then squeezing (Supporting Information Movie S5) through the endothelial barrier [6]. To confirm the rat WBCs were behaving similarly to the endogenous WBCs, we used another transgenic zebra fish line, *Tg(mpeg1:EGFP),* in which GFP is expressed in macrophages of the zebrafish [14]. Macrophages undergo extravasation through diapedesis. The *Tg(mpeg1:EGFP)* line was crossed with the *Tg(fli1a:EGFP)* line, to create a double transgenic zebrafish line where GFP transgene expression was driven in both vasculature and macrophages simultaneously (Fig. 1E). Endogenous WBCs underwent diapedesis (Supporting Information Movie S6) in the same manner as the injected rat WBCs, and within a comparable time frame (Fig. 2F). As a negative control, inert polymer microspheres did not undergo extravasation (Fig. 2G) (Supporting Information Movie S7).

Multiple CSCs Undergo Group Extravasation in a Single Angiopellosis Event

Clusters of cardiac stem cells (CSCs) and mesenchymal stem cells (MSCs) were able to undergo group angiopellosis with the cluster of cells all extravasating simultaneously (Fig. 3A, 3B, 3C). In this case, several cells were lodged in the lumen, with a close proximately to each other. Subsequently, endothelial protrusions encapsulated the entire cell clusters, similar to how they would encapsulate a single CSC/MSC (Supporting Information Movies S8, S9, S16). Once the covering of the cluster was completed, the cluster was deposited outside of the lumen into the surrounding tissue. In contrast, the diapedesis mechanism did not support group extravasation. Clustered WBCs would have one cell leave the cluster and extravasate by itself, or multiple WBCs would leave at the same time (Supporting Information Movie S10), eliciting simultaneous but separate diapedesis events (Fig. 3D).

Angiopellosis Differs Distinctly from Diapedesis

Angiopellosis differs strikingly from diapedesis both morphologically and temporally (Supporting Information Movie S20) [7]. Angiopellosis elicited extensive vasculature activity (i.e., morphological changes of endothelial cells) during the extravasation process, in which endothelial cells actively extended membrane protrusions to encapsulate the foreign cells while remodeling to form an endothelial pockets (Fig. 4A, red line). WBCs did not elicit significant vasculature activity while undergoing the diapedesis process, (i.e., squeeze out without inducing much physical change to the blood vessels) (Fig. 4A, blue line). The roundness of the injected cells during the extravasation process was also quantified. When undergoing angiopellosis, CSCs retained their round shape throughout the whole process (Fig. 4B, red line). In contrast, during diapedesis WBCs underwent extensive morphological change (Fig. 4B, blue line) as they squeeze through the junction through the

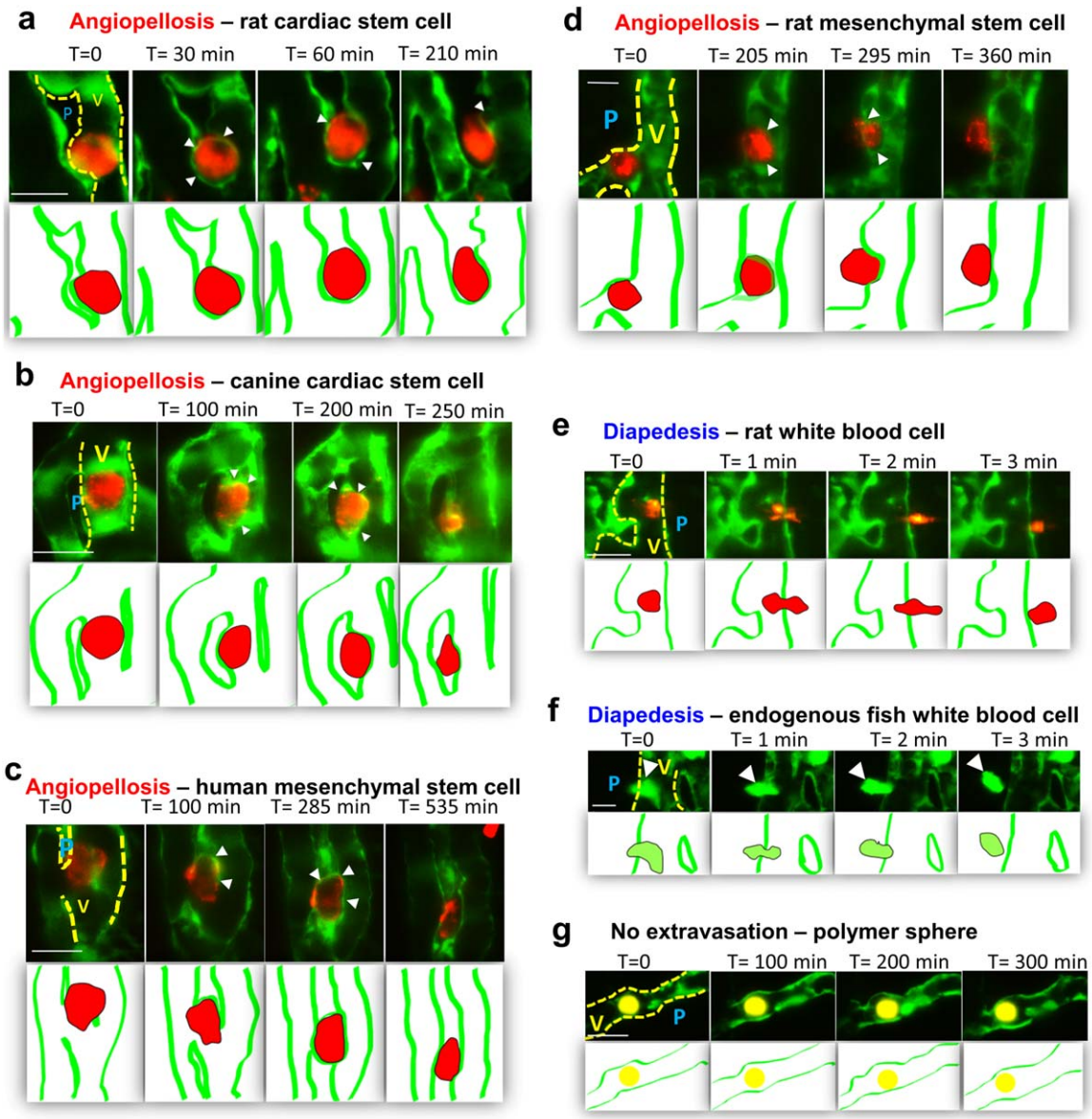

Figure 2. Injected cardiac stem cells undergo extravasation by angiopellosis. **(A)**: Time-lapse imaging in *Tg(fli1a:EGFP)* zebrafish embryos shows intravenously injected rat cardiac stem cell (red) after becoming lodged in the blood vessel (green); T = time in minutes. Gradual protrusions of the vascular endothelial cells can be seen remodeling around the injected stem cell ($T = 30$, arrows). By 3.5 hour the stem cell has completely undergone angiopellosis, and is fully extravasated ($T = 210$). Residual remodeling from the endothelial protrusions are still present and active up until 210 minutes after extravasation ($T = 60$, $T = 210$, arrows). **(B)**: Time-lapse imaging of canine cardiac stem cells (red) extravasating blood vessel (green). Vascular remodeling ($T = 100$, arrows) can be seen actively interacting with the stem cell. Injected stem cell fully extravasates after approximately 250 minutes ($T = 250$). **(C)**: Time-lapse imaging of human mesenchymal stem cells (red) extravasating blood vessel (green). Vascular remodeling ($T = 100$, arrows) can be seen actively interacting with the stem cell. Injected stem cell fully extravasates after approximately 535 minutes ($T = 535$) **(D)**: Time-lapse imaging of human rat stem cells (red) extravasating blood vessel (green). Vascular remodeling ($T = 205$, arrows) can be seen actively interacting with the stem cell. Injected stem cell fully extravasates after approximately 6 hours ($T = 360$). **(E)**: Time-lapse imaging in *Tg(fli1a:EGFP)* zebrafish embryos shows intravenously injected rat white blood cell (red) undergoing diapedesis to extravasate out the zebrafish blood vessel (green). **(F)**: Time-lapse imaging in double transgenic *Tg(fli1a:EGFP)/(mpeg1:EGFP)*, in which both vasculature and endogenous macrophages express GFP. Zebrafish embryos endogenous white blood cell (arrow) undergo diapedesis to extravasate blood vessel in characteristic manner. **(G)**: Time-lapse imaging in *Tg(fli1a:EGFP)* zebrafish embryos shows intravenously inert 8 μm polymer microspheres (yellow), the microsphere remains in the relatively same position over the course of approximately 300 minutes. Yellow dotted line represents the vascular wall of the blood vessel. V = vasculature lumen; P = parenchymal surrounding tissue. All scale bars = 20 μm.

endothelial cells. Control polymer microspheres retained their roundness throughout the entire time they were observed (Fig. 4B, black line). The extravasation efficiencies for angiopellosis or diapedesis were similar (Fig. 4C), although angiopellosis took significantly longer to occur as opposed to the relatively quick diapedesis process (Fig. 4D).

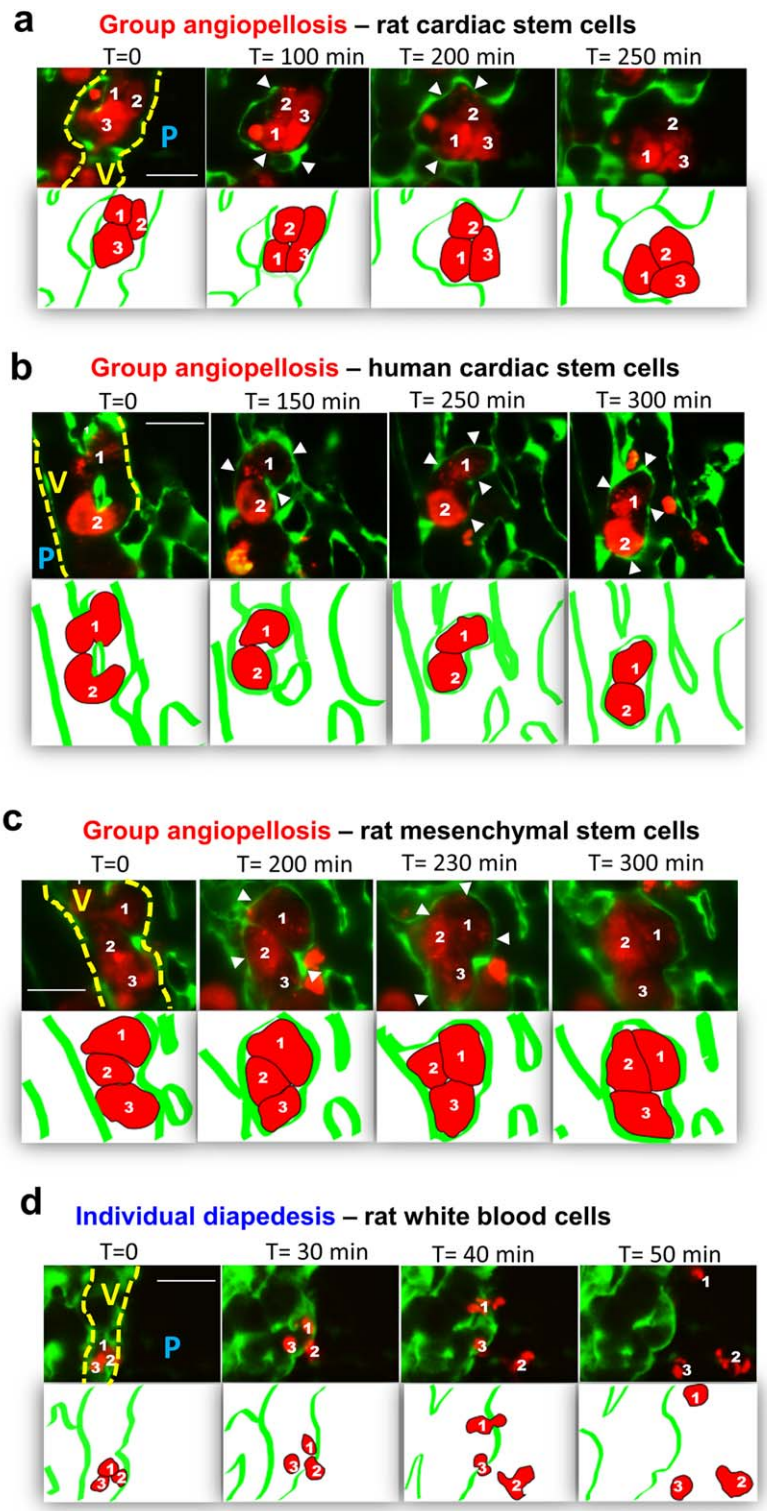

Figure 3. Multiple CSCs undergo cluster extravasation in a single angiopellosis event. **(A)**: Time-lapse imaging in *Tg(fli1a:EGFP)* zebrafish embryos shows intravenously (IV) injected rat cardiac stem cell (red) cluster lodged in the blood vessel (green); T = time in minutes. Clustered cells underwent extravasation with endothelial protrusions (arrows) extending around three injected cells, prompting vascular remodeling. **(B)**: Time-lapse imaging shows IV injected human cardiac stem cell cluster; formation of endothelial protrusions occurs around two cells ($T = 250$, arrows). This is proceeded by the injected cells exiting the blood vessel lumen into an intervascular pocket/cavity ($T = 300$, arrows). **(C)**: Time-lapse imaging shows IV injected rat mesenchymal stem cell cluster; formation of endothelial protrusions occurs around three cells ($T = 200$, arrows). This is proceeded by the injected cells exiting the blood vessel lumen into an intervascular pocket/cavity ($T = 300$). **(D)**: Time-lapse imaging shows IV injected rat white blood cell cluster; cells exit lumen through diapedesis within 1 hour ($T = 50$). Yellow dotted line represents the vascular wall of the blood vessel. V = vasculature lumen; P = parenchymal surrounding tissue. All scale bars =20 μm. Abbreviations: CSCs, cardiac stem cells; MSCs, mesenchymal stem cells.

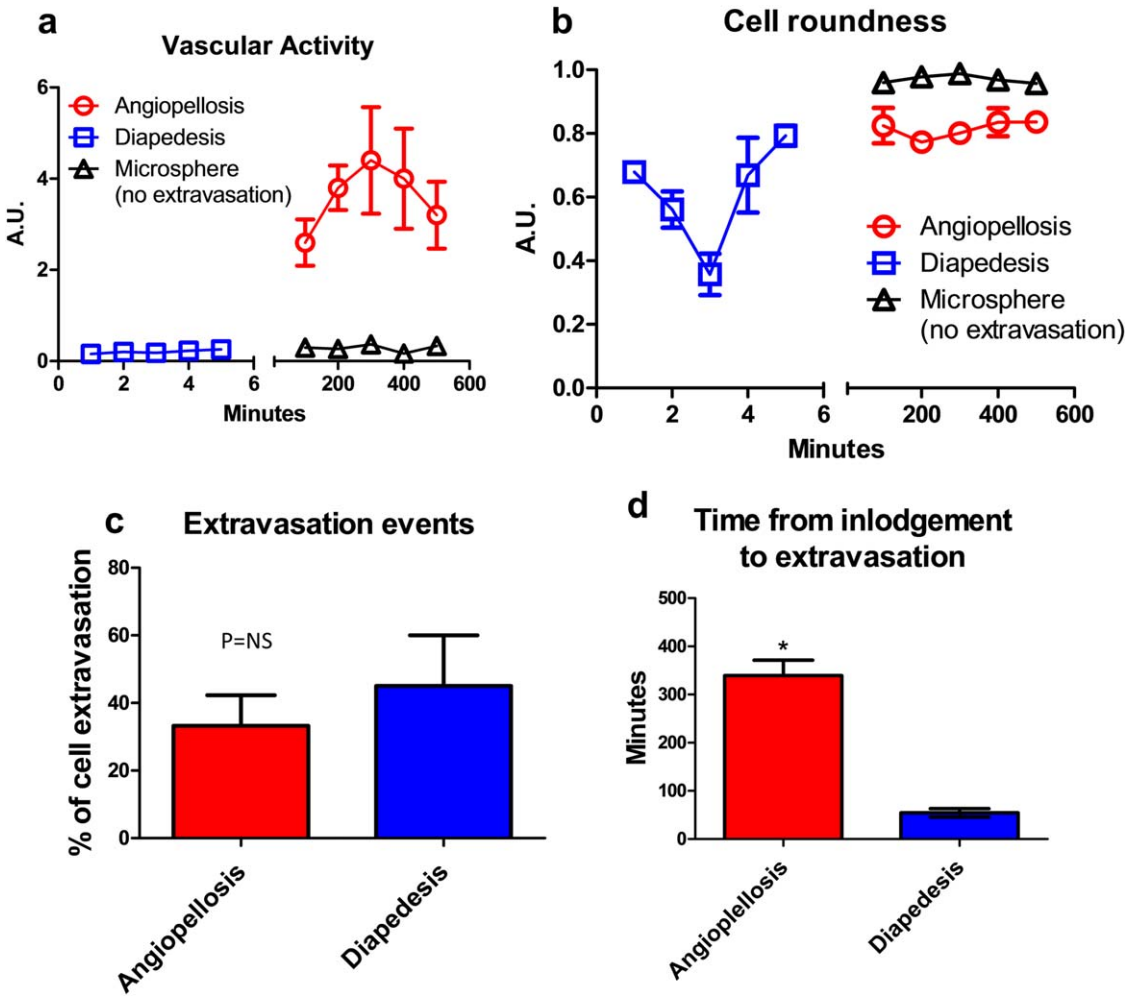

Figure 4. Morphological and temporal differences between angiopellosis and diapedesis. **(A)**: Quantification of the change in vascular activity (endothelial cell movement) during extravasation of injected cells was averaged from all respective extravasation events. Injected stem cells (red, $n = 40$ injected cells, $N = 10$ zebrafish) prompted an increase in vascular activity during the angiopellosis event, with levels peaking mid-process and returning to baseline after the completion of the extravasation. White blood cells (blue, $n = 10$ injected cells, $N = 3$ zebrafish) did not elicit significant vascular activity as they pass through the blood vessel while the endothelial cells remain mostly passive. Polymer microspheres (black, $n = 10$ injected spheres, $N = 3$ zebrafish), similarly did not elicit significant vascular activity and no extravasation happened. **(B)**: Quantification of the roundness of injected cells during extravasation events. Injected stem cells (red, $n = 40$ injected cells, $n = 10$ zebrafish) remained round in morphology during the angiopellosis process. White blood cells (blue, $n = 10$ injected cells, $n = 3$ zebrafish) lost round shape as they squeezed through the endothelial cells, and returned to a more round shape once outside of the blood vessel. Polymer microspheres (black, $n = 10$ injected spheres, $n = 3$ zebrafish) remained round but did not extravasate. **(C)**: Percentage of either type of extravasation events was not significant. Angiopellosis data (red) was obtained from all zebrafish injected with CSCs ($N = 50$ zebrafish) from different species and averaged together. Diapedesis data (blue) was obtained from the averaging all events of extravasation observed ($n = 3$). **(D)**: The time required for injected stem cells to extravasate through angiopellosis (red, $n = 40$ extravasating cells, $N = 10$ zebrafish) after becoming lodged in vasculature was significantly longer than diapedesis of WBCs (blue, $n = 10$ extravasating cells, $N = 3$ zebrafish). Asterisk: $p < .05$ by two-tailed Student's *t*-test.

Polymer Microspheres Coated with Cell Membrane Can Undergo Extravasation by Angiopellosis

As shown in Fig. 2E, inert polymer spheres could not undergo extravasation. We then sought to determine if the angiopellosis type of extravasation was prompted by cell-cell interaction between the injected cells and the endothelial cells since polymer microspheres did not contain the cell membrane ligands as CSCs did. To test this hypothesis, we cloaked microspheres with rat CSC membrane (Fig. 5A). Fluorescent microscopy was used to confirm the efficient membrane cloaking onto the spheres (Fig. 5B). We then injected the coated microspheres into the blood stream of zebrafish and observed extravasation events *in vivo* using lightsheet microscopy. Unlike un-coated spheres (Fig. 5C) (Supporting Information Movie S11), CSC membrane-coated polymer spheres were observed to undergo extravasation by angiopellosis (Fig. 5D) (Supporting Information Movie S12). The percentage of coated spheres that extravasated was similar to the amount of CSCs that underwent extravasation when injected (Fig. 5E).

Angiopellosis Does Not Depend on the Same Membrane Molecule Interaction as Diapedesis

To determine if angiopellosis extravasation was dependent on the same membrane molecule interaction as diapedesis, we

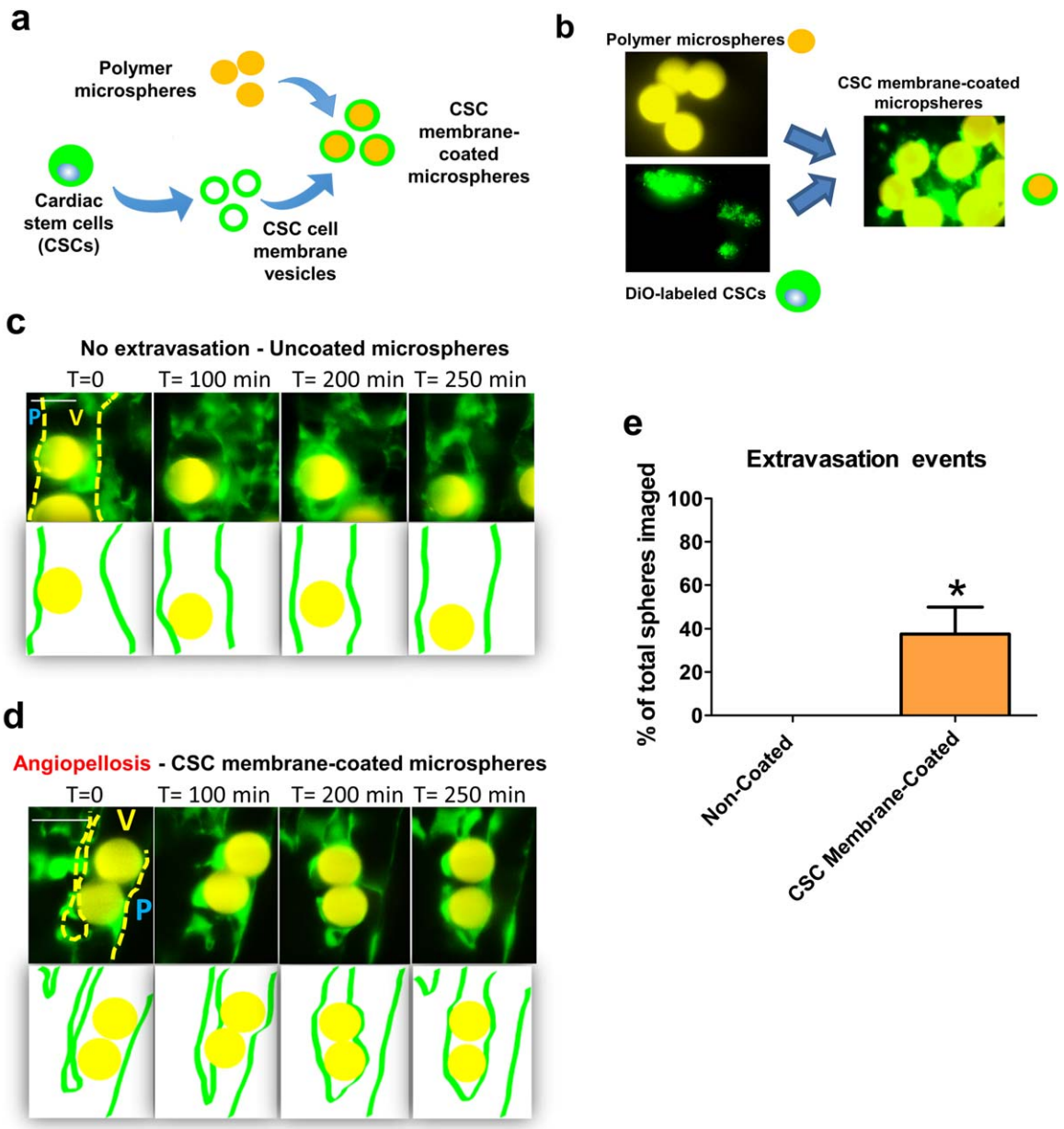

Figure 5. Polymer microspheres coated with cell membrane can undergo extravasation by angiopellosis. **(A, B)**: Polymer microspheres were coated with the membranes of CSCs to create CSC membrane-coated microspheres. These microspheres were then injected intravenously into the zebrafish (48 hpf) and were observed for extravasation events. Successful membrane coating was confirmed using fluorescence microscopy. **(C)**: Uncoated microspheres (yellow) moved through the blood vessels but did not extravasate at all. Scale bar = 20 μm. V = vasculature lumen; P = parenchymal surrounding tissue. **(D)**: CSC membrane-coated microspheres were injected and observed to undergo extravasation through angiopellosis, in the same manner as the CSCs; scale bar 20 μm. **(E)**: The percentage of extravasation for both CSC membrane coated ($n = 3$) and uncoated ($n = 10$) microspheres was quantified. Asterisk: $p < .05$, two-tailed Student's *t*-test. Abbreviations: CSCs, cardiac stem cells.

inhibited the integrin CD11α of the injected cells prior to IV infusion. CD11α is an integrin that mediates cell adhesion and extravasation in diapedesis. Cardiac (Fig. 6A) and mesenchymal (Fig. 6B) stem cells retained the ability to undergo angiopellosis when CD11α was inhibited. Both single and cluster angiopellosis events were observed with CD11α inhibition (Supporting Information Movies S14, S15). In contrast, injected white blood cells (Fig. 6C) lost the ability to undergo diapedesis when CD11α was inhibited (Supporting Information Movies S17, S18). The percentage of CD11α-inhibited injected stem cells that were able to undergo angiopellosis remained similar to that of uninhibited cells, while the CD11α inhibition effectively prevented all WBCs from extravasating (Fig. 6D).

Discussion

Our study uncovered a previously unreported mechanism of cell extravasation, which is distinct from the well characterized diapedesis process. Angiopellosis sheds light on the

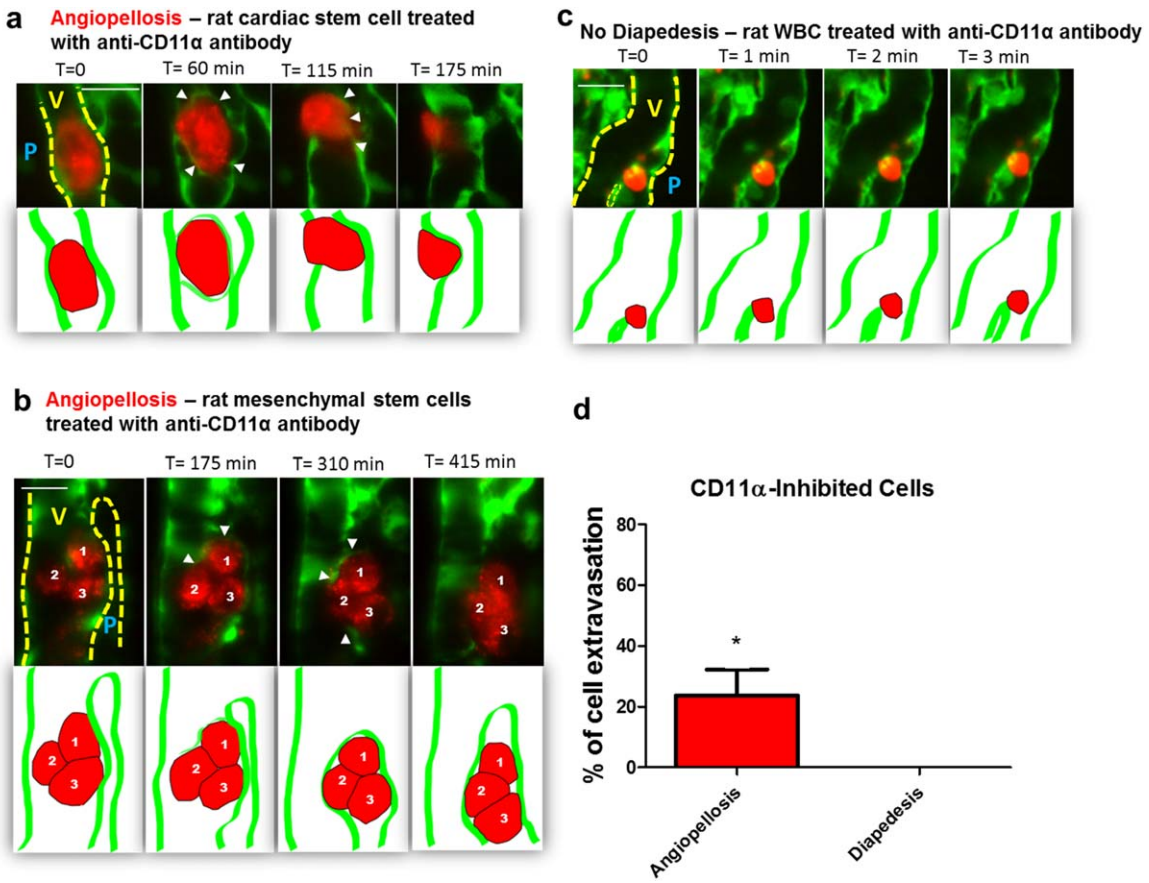

Figure 6. Angiopellosis is not dependent on integrin CD11α. **(A)**: Time-lapse imaging in *Tg(fli1a:EGFP)* zebrafish embryos shows intravenously injected rat cardiac stem cell (red) pretreated with anti-CD11α antibodies. Images show cells after becoming lodged in the blood vessel (green); T = time in minutes. Gradual protrusions of the vascular endothelial cells can be seen remodeling around the injected stem cell ($T = 60$, arrows). By 3 hours the stem cell has completely undergone angiopellosis, and is fully extravasated ($T = 175$). **(B)**: Time-lapse imaging in *Tg(fli1a:EGFP)* zebrafish embryos shows intravenously (IV) injected rat mesenchymal stem cell (red) cluster pretreated with anti-CD11α antibodies. Images show cells lodged in the blood vessel (green); T = time in minutes. Clustered cells underwent extravasation with endothelial protrusions (arrows) extending around three injected cells, prompting vascular remodeling. **(C)**: White blood cells (red) pretreated with anti-CD11α antibodies migrated inside the blood vessels but did not extravasate at all. Scale bar = 20 μm. V = vasculature lumen; P = parenchymal surrounding tissue. **(D)**: The percentage of both angiopellosis of stem cells and diapedesis of WBCs I.V. injected after treatment with anti- CD11α antibodies was quantified. Angiopellosis data (red) was obtained from all zebrafish injected with CSCs and MSCs ($N = 4$ zebrafish) from different species and averaged together. Diapedesis data was obtained from the averaging all events of extravasation observed ($n = 8$ zebrafish). Scale bar = 20 μm. Asterisk: $p < .05$, two-tailed Student's t-test.

actual mechanism intravenously injected cells, which are not specialized for extravasation, use to exit the blood vessel lumen (Fig. 1A). The ability of this extravasation process to happen with cells injected from varying species (Fig. 2A, 2B; Fig. 3A, 3B) into the zebrafish model suggests a well conserved mechanism, which has been retained evolutionarily. The known similarity of genes involved in vasculature activity between zebrafish and mammals suggests this mechanism of extravasation also occurs in mammals [15]. Similarly, diapedesis was also observed with WBC introduced from a different species in the foreign zebrafish model (Fig. 2E). This further represents the ability of WBCs to extravasate the blood system without an immune or inflammatory response being present, and also reinforces the notion that WBC motility is retained when introduced to different systems/species [8]. Since both foreign stem cells (Fig. 1A–1C) and foreign WBCs (Fig. 2E) are able to extravasate from the fish blood stream, it can be concluded that an interaction within the blood vessel must prompt cells to exit the lumen if it is not advantageous for the cells to remain inside [16].

Once prompted to extravasate, the ability of injected stem cells to remain relatively round in morphology (Fig. 4B) while undergoing angiopellosis represents their ability to passively leave the blood vessel. Since the majority of the morphology change occurs within the endothelial cells of the blood vessel (Fig. 4A), it signifies the blood vessels themselves are actively removing the foreign cells from the lumen [17]. In contrast to angiopellosis, when WBCs undergo diapedesis, they have distinct morphological changes to form a stretched cell shape (Fig. 2E, 2F) to squeeze tightly through the endothelial cells of the vessel wall [6]. In contrast, angiopellosis utilizes the endothelial cell's ability to restructure, allowing the injected cells to only undergo a minimal amount of visible alteration to exit the blood vessel [17]. This key feature is likely what allows for the wide range of extravasation event to occur across different cells are not specialized for blood vessel transmigration.

Angiopellosis supports group extravasation while diapedesis does not. Unlike diapedesis, in which one white blood cell leaves per diapedesis event [6, 18] (Fig. 3C), the angiopellosis process allows for several cells to leave simultaneously during a single angiopellosis event (Fig. 3A, 3B). This could be caused by the close proximity of the injected cells to one another, or perhaps the endothelial cells not being able to distinguish a cluster of cells from a single cell. Since it is the endothelial cells of the blood vessel that are actively being remodeled, it could potentially not matter the number of cells that need removal, as long of they are close enough to become encapsulated by the endothelial protrusions. Tumor cells have been reported to have increased metastatic potential when circulating in clusters, and angiopellosis could present a potential method these clusters use to extravasate and invade distant tissues [19–21].

Strong temporal differences in the angiopellosis and diapedesis mechanisms further assist in the contrasting of their differences. Once the injected cells become lodged in the blood vessel lumen, the endothelial protrusions can take hours (Fig. 4D) to completely form around the cell(s) before the injected cells can be removed from the lumen. Certain intravenously infused stem cells are known to home in on damaged tissue and seemingly activity seek out the site of the injury [22, 23]. The site of injury could be exuding signals that are recognized by the injected stem cells, which prompts them to attach to the blood vessel wall, which then stimulates the angiopellosis process to occur. Chemokines and their receptors have been reported as important mediators in the process of stem cell homing [24]. Another possibility is that signals on the injected cell's membrane are interacting with the receptors on the blood vessel endothelial cells, asking for the cells removal from the lumen. As a control, inert polymer microspheres did not elicit angiopellosis or extravasation (Fig. 5C). However, when the same microspheres were coated with the membrane of CSCs they could undergo extravasation by angiopellosis (Fig. 5D), suggesting angiopellosis relies on biological recognition on the cell membrane.

When integrin CD11α is inhibited with an anti-CD11α antibody, WBCs (Fig. 6C) lose their ability to extravasate (Supporting Information Movie S18), while stem cells (Fig. 6A, 6B) are still able to undergo extravasation by angiopellosis. CD11α is essential for cellular rolling and adhesion during diapedesis [11]. Interestingly, even after being stripped of their diapedesis ability WBCs were not able to utilize the angiopellosis mechanism to extravasate, suggesting that the mechanism of cellular recognition and endothelial wall attachment used during angiopellosis differs from that during diapedesis. These observations, coupled with the ability of microspheres coated with CSC membranes to angiopellose, delivers striking evidence that although cellular membrane recognition is involved in angiopellosis, the membrane molecules involved in angiopellosis are distinctly different from those involved in diapedesis. We propose that the endothelial cells of the blood vessel recognize a biological/cellular component(s) specific to certain cell types foreign to the blood stream, and prompts the angiopellosis event to occur. These cellular molecules could be exclusive to certain cell types (e.g., stem and cancer cells), which would account for the inability of native red blood cells, and injected CD11α-inhibited WBCs from utilizing the angiopellosis mechanism to extravasate. Or perhaps, cells native to the blood stream have molecules/signals that serve as angiopellosis inhibitors, which prevents the attachment and vascular coating steps of angiopellosis.

Table 1. Differences between Angiopellosis and Diapedesis extravasation

	Angiopellosis	Diapedesis
Time required for extravasation	Hours	Minutes
Cell roundness during extravasation	High	Low
Group extravasation	Yes	No
Vascular activity during extravasation	High	Low
Ability to extravasate when integrin CD11α is inhibited	Yes	No

Table summarizing the main differences between the angiopellosis and diapedesis methods of cell extravasation. Time required for extravasation is the approximate amount of time cells required to fully undergo the respective extravasation events. Cell roundness during extravasation is refers to the roundness of cell during the respective extravasation events. Group extravasation refers to the potential of groups/clusters of cells to leave the blood vessel in a single extravasation. Vascular activity during extravasation refers to the amount of change in position and structure in the endothelial cells of the blood vessel during the respective extravasation events. Angiopellosis is still able to occur when CD11α is inhibited, while CD11α inhibition effectively prevents white blood cells from undergoing diapedesis.

The present work reported and characterized angiopellosis as a cell extravasation mechanism distinct from the conventional diapedesis (Table 1). Future directions for this research will involve elucidating the exact cellular signals/molecules required for angiopellosis initiation [25]. Once these signals/pathways are identified, we can then delve further into ways to modify the pathways and alter the ability of intravenously injected cells to exert their regenerative effects; this mechanism could also have implications for how cancer cells, and circulating tumor clusters extravasate and metastasize [26–28].

Methods

Animals

All experiments involving live zebrafish and rats were performed in accordance with relevant institutional and national guidelines and regulations and were approved by the North Carolina State University Institutional Animal Care and Use Committee. The transgenic lines *Tg(fli1a:EGFP)* and *Tg(mpeg1:EGFP)* were used in this study [14, 29]. For the creation of the double transgenic *Tg(fli1a:EGFP)/(mpeg1:EGFP),* male *Tg(fli1a:EGFP)* individuals were mated with female *Tg(mpeg1:EGFP)* individuals. The resulting embryos were screened at 48 hpf for the expression of both transgenes, and double transgenic embryos were collected. In order to prevent pigmentation, 0.2 mM N-phenylthiourea (PTU; Sigma) was applied to embryos starting at 24 hpf.

Isolation and Culture of Cardiac Stem Cells and Mesenchymal Stem Cells

Human, rat, and canine cardiac stem cells (CSCs) were derived from human endomyocardial biopsies, Wistar-Kyoto rat hearts, and adult male canine hearts respectively. CSCs were generated using the cardiosphere method as previously described [28]. Rat adipose-derived mesenchymal stem cells (MSCs)

were derived from the same strain of rats as previously described and human adipose-derived MSC were derived using the previously published method [30]. Derived CSCs and MSCs were cultured in IMDM with 20% FBS media and passaged every 3–5 days.

Embryo Preparation and Cell Implantation

Dechorionized 48 hpf zebrafish embryos were anaesthetized with 0.004% tricaine (Sigma) and positioned on a 200 × 15 mm Petri dish coated with 3% agarose. Mammalian cells were trypsinized into single cell suspensions, resuspended in phosphate-buffered saline (PBS; Invitrogen), kept at room temperature before implantation and implanted within 2 hours. Cells were labeled with the fluorescent cell tracker DiI (Invitrogen) according to the manufacturer's instructions. The cell suspension was loaded into borosilicate glass capillary needles (1 mm o.d. × 0.78 mm i.d.; World Precision Instruments) and the injections were performed using a PV830 Pneumatic Pico pump and a manipulator (WPI). 50–100 cells, or 10–15 μm microspheres (Bangs Laboratory, Fishers, Indiana, http://www.bangslabs.com/), were injected at approximately 50 μM above the ventral end of the duct of Cuvier where it opens into the heart. The approximate injection parameters were: injection pressure = 300 p.s.i., holding pressure = 10 p.s.i., injection time = 0.2 ms. Injected mammalian cells could normally be seen entering the vasculature 15–30 minutes after injection and starting to arrest in the TAVs 1–2 hours after injection. After implantation with mammalian cells, zebrafish embryos (including non-implanted controls) were maintained at 28°C. Normally, cell injected embryos were euthanized at the end of experiments (~72 hpf) by tricaine overdose. For each cell line or condition, data are representative of ≥ three independent experiments, with ≥5 embryos/group. Experiments were discarded when the survival rate of the control group was < 80%.

White Blood Cell (Leukocyte) Isolation

White blood cells were isolated from fresh whole blood samples. Sprague Dawley Crl:SD (Charles River) rats were used for the collection of < 5 ml of whole blood. Briefly, the whole blood was transferred into a new 9 ml EDTA-treated tubes (Greiner-Bio-One), to prevent coagulation. The whole blood was layered onto histopaque 1083 (Sigma) and centrifuged at 1,700 rpm for 30 minutes. The mononuclear cell layer was recovered and spun at 1,300 rpm for 10 minutes. Cells were labeled with the fluorescent cell tracker DiI (Invitrogen) according to the manufacturer's instructions. Cells were injected within 2 hours after isolation.

Zebrafish Preparation and Microscopy

For live imaging in the light-sheet microscope, 48 hpf zebrafish embryos were anaesthetized using 0.016% tricaine (Sigma) and then were embedded in 1.3% low-melting-temperature agarose (Sigma; prepared in filtered fish facility water) inside a glass capillary (1.5/2.0-mm inner/outer diameter, 20-mm length (Zeiss). The larvae were centered in the capillary and oriented. After gel formation, the section of the agarose cylinder containing the tail of the embryo was extruded from the capillary by inserting wax into the capillary on the side opposite to the fish. The sample chamber of the light-sheet microscope was filled with filtered fish facility water, and the capillary was inserted for imaging. Specimens were maintained at 28°C throughout the imaging period. Fluorescent image acquisition was performed using a Zeiss Lightsheet Z.1. Z-stacks were processed for maximum intensity projections with Zeiss ZEN software. For timelapse (4D) images, z-stacks were taken every 5–15 minutes for a total time of up to 24 hours with a step number between 50 and 200 and step size of 0.3–2.0 μm. Images were adjusted for brightness and contrast using Zeiss ZEN Software. Confirmation of injected cell migration from inside of the lumen to surrounding tissue was done using the Zeiss ZEN software 3D retendering capability (Supporting Information Movie S13). Cell roundness and vascular activity was measured using ImageJ software.

Creation of Cell Membrane-Coated Microspheres

FluoSphere Polystyrene microspheres (Life Technologies) were used. To prepare the cardiac stem cell (CSC) membrane-coated microspheres, DiO (Sigma)-labeled CSCs went through three freeze/thaw cycles. After which, the disrupted CSCs were sonicated for approximately 5 minutes at room temperature along with the microspheres. After that, the microspheres were washed three times in PBS by centrifugation. Successful membrane coating was confirmed using fluorescence microscopy.

Pretreatment of Injected Cells with anti-CD11α Antibodies

Rat cardiac stem cells and rat adipose-derived MSCs were suspended in 5 ml of cell media, and were treated with 5 μl of Biolegend Purified anti-rat CD11a Antibody (Concentration: 0.5 mg/ml) for 1.5 hours. Cells were then labeled with the fluorescent cell tracker DiI (Invitrogen) according to the manufacturer's instructions. White blood cells were isolated from fresh whole blood samples as previously stated. WBCs were suspended in 5 ml of cell media, and were treated with 5 μl of Biolegend Purified anti-rat CD11a Antibody (Concentration: 0.5 mg/ml) for 1.5 hours. Cells were then immediately injected into embryos as stated previously. The antibody recognizes both activated and unactivated LFA-1 and inhibits the binding of LFA-1 to ICAM-1. The antibody has been shown to inhibit the mixed lymphocyte reaction, leukocyte infiltration, and graft rejection [11].

Statistical Analysis

All statistical analysis was performed using Graphpad Prism 5 (Graphpad Software, La Jolla, CA). Two-sided tests were performed for all analyses; ns, not significant ($p > .05$); $p < .05$ was considered significant and indicated with a single asterisk, and error bars represent standard deviation. Comparisons between two groups were performed using a student's *t*-test.

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Author Contributions

T.A., K.C. conceived of and designed the experiments. T.A., D.G., M.T., D.A.T., K.C. performed the experiments. M.T.H, J.C, A.V, J.T, J.B.M.A, P.D., J.A.Y. analyzed and interpreted the results. T.A wrote the manuscript. K.C. directed the project and revised the manuscript.

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