Disruption of Angiopoietin-1 and $\alpha_v\beta_3$ Integrin Interaction in Tie2, Ang-1 and $\alpha_v\beta_3$ Integrin complexes

Bold: primary proteins that are under investigations, or experiment steps

Italic: words that have definition or extra information in Glossary section

Introduction:

Cancer is the second leading cause of death worldwide. Estimation of 9.6 million deaths globally, 6 hundred-thousand deaths and 1.7 million new cases diagnosed in the US in 2018. [1,2] At some point in their lives, 38.4% of the population will be diagnosed with cancer. Cancer care expenditures reached $147.3 billion in 2017.[2]

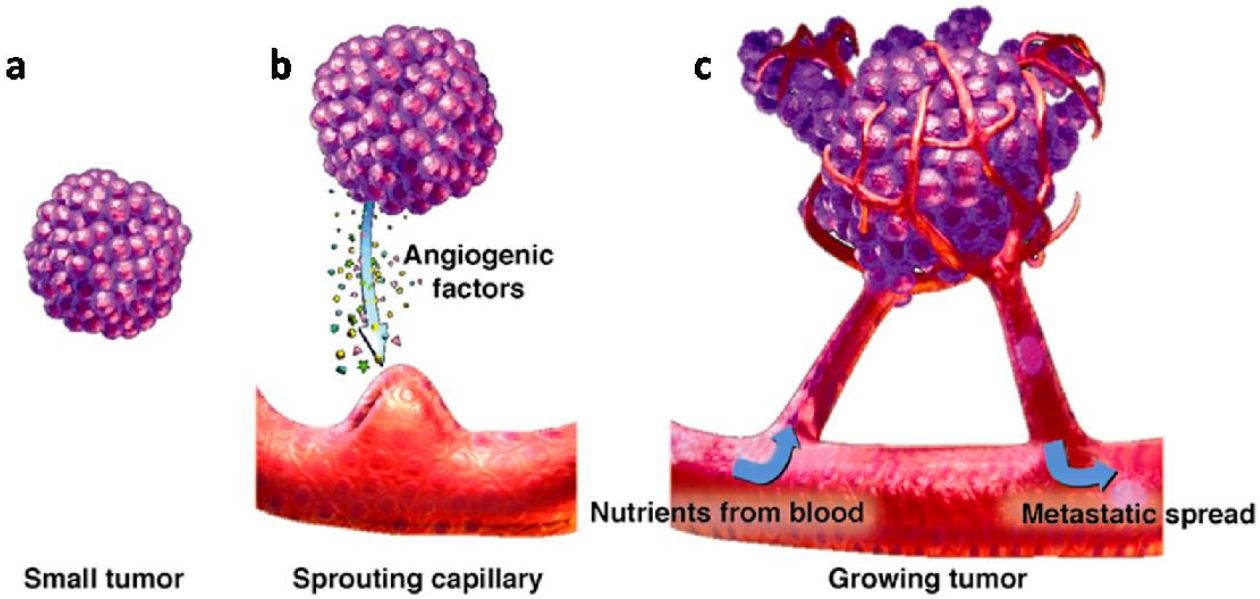


Fig.1. Angiogenesis in tumor cells [3]

Cancer is a disease when cells in one's own body have gone wrong. In the early development of cancer, a clump of cancer cells is limited by diffusion and cannot grow larger than a certain size. Absorption of nutrients from the environment and waste removal through their surface area are no longer sufficient. A different mass movement mechanism is required (**Fig.1**): inducing and maintaining blood and lymph vessels grown from nearby vessels (*angiogenesis*) and connected to the host circulation system [4]. An extensive molecular knowledge of main angiogenesis pathways is desired to control cancer angiogenesis, ultimately to limit the growth of tumor.

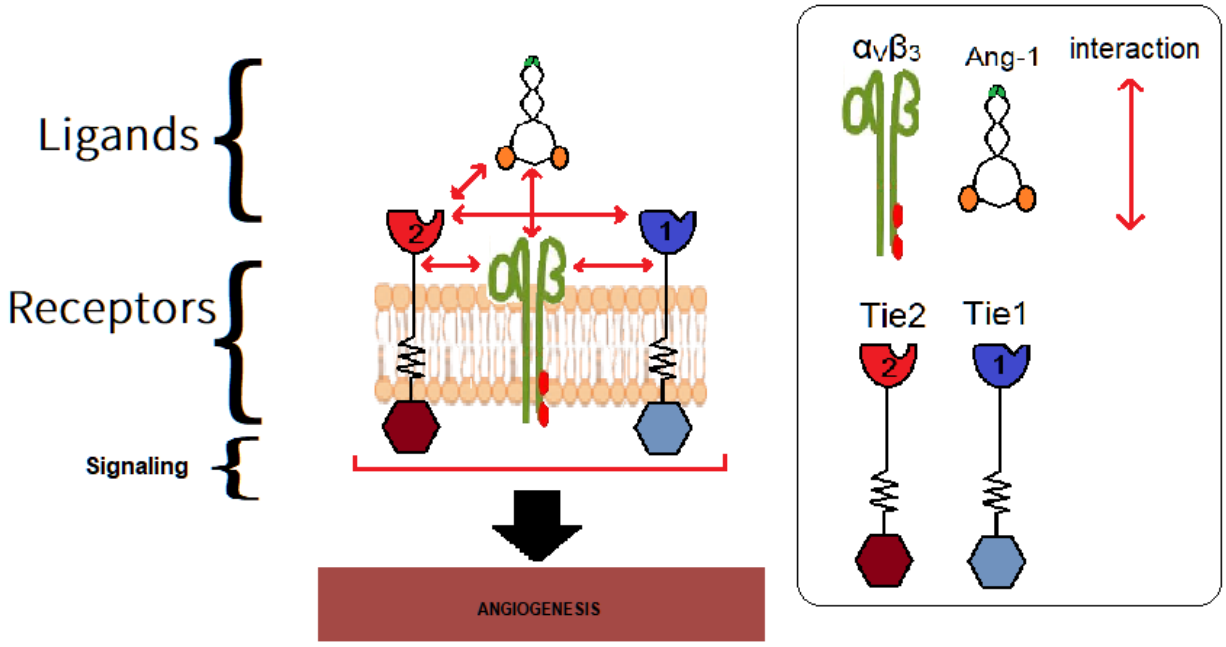


Fig.2. Schematic diagram of main angiogenesis pathways. Redrawn from ,[5] with added information from.[6]

Tie receptors and their ligands *angiopoietins* play a crucial role in regulating tumor-induced angiogenesis (**Fig.2**).[7] **The *receptor tyrosine kinase* Tie2 (RTK-Tie2)** is a transmembrane receptor protein. High expression of Tie2 receptor in *endothelial* cells is crucial for angiogenesis and vessel maintenance .[8] **Angiopoietin-1, one** of 4 Tie2 protein ligands (Ang1-4), has been recognized as the primary activating ligand for Tie2, and is a constitutive receptor *agonist.* [(8,9)] In endothelial cells, activation of Tie2 by binding of Ang-1 at the *ectodomain* causes auto-phosphorylation, which subsequently leads to the activation of a number of intracellular signaling pathways; ultimately resulting in endothelial cell migration ,[(10,11)] tube formation ,[12] sprouting [13] and cell survival [11]. **Tie1**, is a transmembrane protein and homologous to Tie2, however it does not directly interact with any angiopoietins [14]. Studies in mice shows that embryos that lack Tie1 usually suffer *pulmonary edema*, vessel integrity defects, and localized hemorrhaging, and die *in utero*. Tie1 is speculated to play a role in vessel integrity. However, the exact role of Tie1 in angiogenesis is still unknown.

Studies have shown that there is an association between Tie1 and Tie2 on the cell surface, prior to ligand recognition .[15] In the presence of Tie1, Tie2 basal activation is drastically decreased (~50% compared to no Tie1) .[15] Ang1 and Ang2 exhibit different roles when interacting with the Tie1/Tie2 complex. **Ang-2** when bound to the *heterodimer* complex exhibits an antagonistic characteristic (**Fig.3.C**), compared to an agonist role without the presence of Tie 1 (**Fig.3.A**). The binding of Ang-1 to Tie2 in the heterodimer complex however promotes *heterodimer dissociation*, forming Tie2 cluster, and signaling initiation (**Fig.3.B**) *[15].*

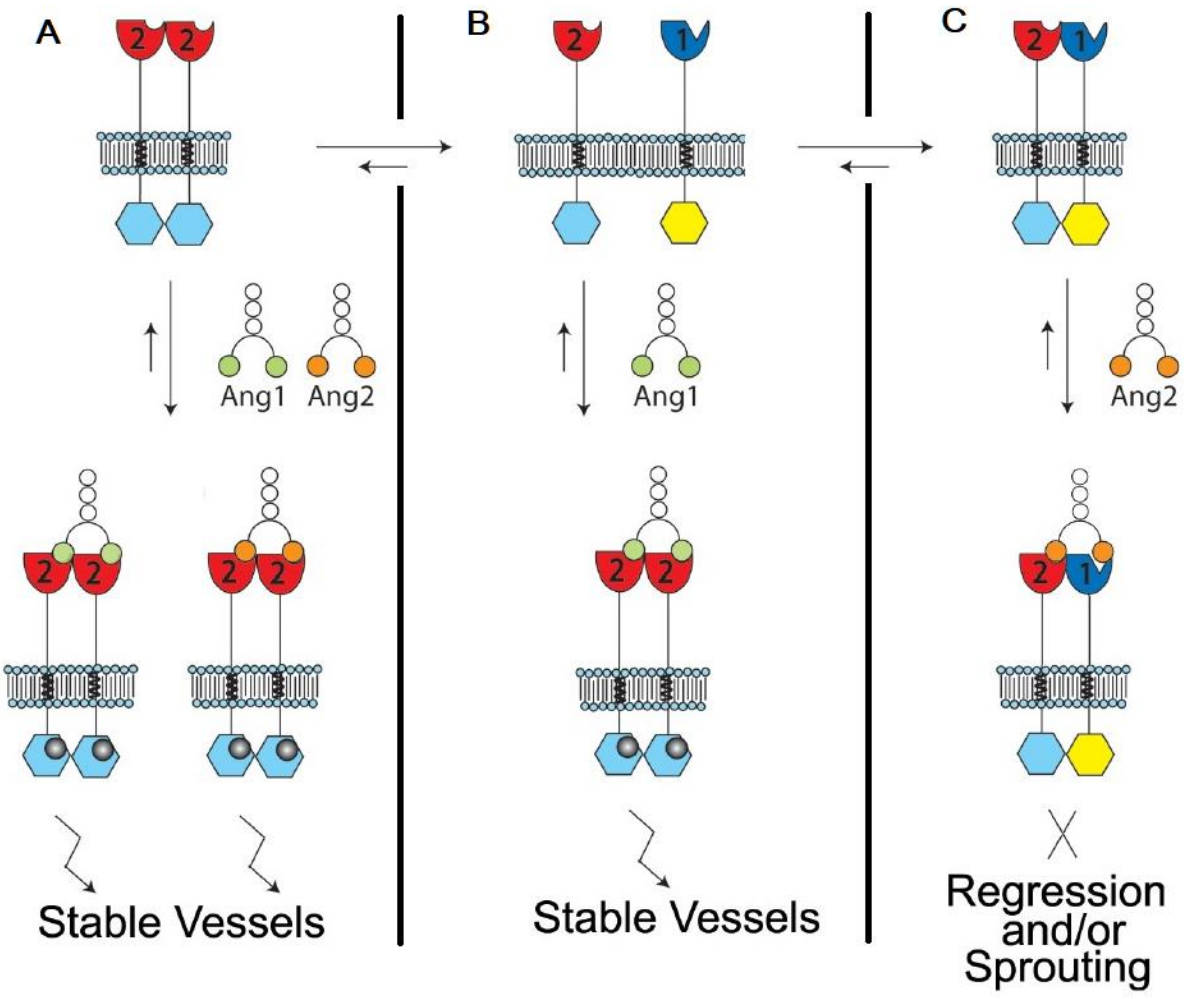


Fig.3. Model for Angiopoietin-mediated Tie2 signaling [15]

The Ang-Tie system certainly offers an important drug target to treat tumor:

- Drugs that are broad-spectrum inhibitors of RTKs (including Tie2): [16,17] Cabozantinib, Lenalidomide, Regorafenib…
- Drug that is inhibiting Angiopoietin 1 & 2 interaction with receptor Tie2 [18]: Trebananib.

Compound treatments target different pathways and interactions. Therefore, it is necessary to study and explore different potential drug targets.

Recent studies have revealed an association between Ang-Tie system and the Integrins. Tie1 and Tie2 freely interact with the **Integrins** $\alpha_5\beta_1$ and $\boldsymbol{\alpha_V\beta_3}$ in a mutually exclusive manner. through their ectodomains (i.e. they are competing for binding with integrin (**Fig.5.row5.column 1 and 2**).[6] The Integrins are membrane protein, that link actin inside the cell to the matrix protein outside of the cell .[19]

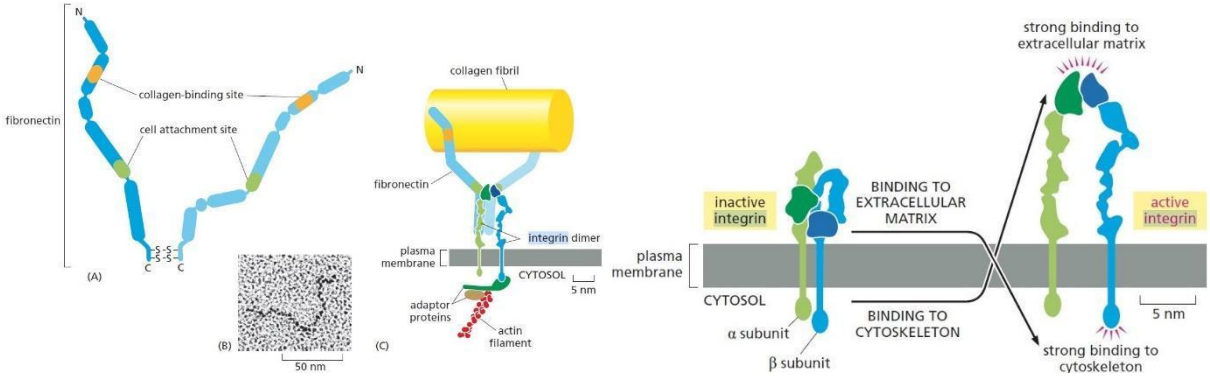


Fig.3.1. Integrins functions and different conformations [19]

Integrin-Tie recognition may help split up the Tie1/Tie2 complex [6]. Tie-integrin recognition is direct and independent of the presence of Ang ligands, only the Tie2 ligand Ang-1 receptor binding domain can interact with $\alpha_5\beta_1$ and $\alpha_V\beta_3$.[6] Association of the Tie2-integrin complex is dramatically enhanced in the presence of the extracellular component and integrin ligand fibronectin. Furthermore, Ang1-RBD-Fc (receptor-binding domain-fragment crystallizable) has been shown to be also capable of independent binding to integrin proteins (**Fig.5.row 5.colum 3**) regardless of the presence of Tie2.[6] Association with Integrin increases the sensitivity of Tie2 to Ang1 (**Fig.5.row 6**).[6]

Studies have also suggested a function for angiopoietin ligands that are dependent on integrin but independent of Tie2. [20-22] Angiopoietin-induced effects also have been described in *cardiomyocytes*, breast cancer cells, and neurons, cells in which Tie2 is not expressed. [23-27]

This proposal explores the potential of disrupting Integrin ($\alpha_V\beta_3$) and Ang-1 association in altering and potentially (speculatively) lowering Tie2 autophosphorylation. This proposal uses an *antibody* to disrupt the association between Integrin ($\alpha_V\beta_3$) and Ang-1, while interaction between Ang-1 and Tie2 remain the same (**Fig.7. and Fig.8**). If autophosphorylation of Tie2 is turned down indirectly by disruption caused by antibody on Integrin ($\alpha_V\beta_3$) and Ang-1 interaction, their association can be a potential drug target.

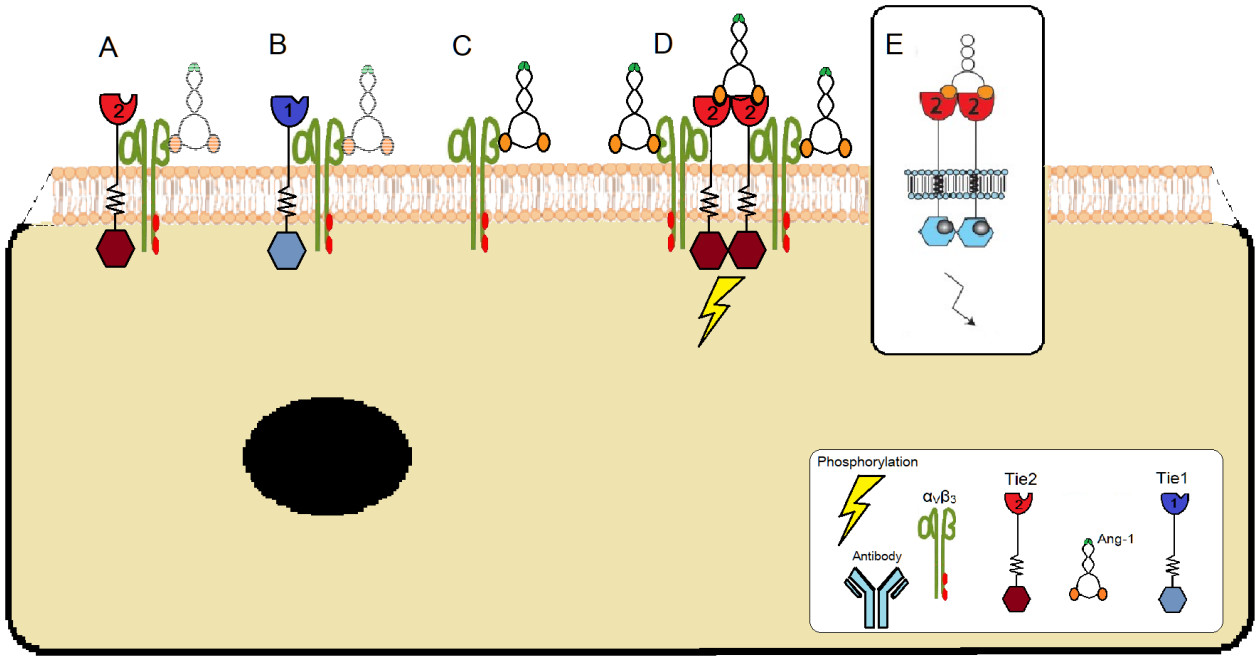


Fig.4. Model for associations of Tie1, Tie2, and Ang-1 with αVβ3 integrin.

(A) Tie2 does not affect ability of Ang-1 to bind to $\alpha_V\beta_3$

(A, B) Ang ligands do not affect Tie/integrin interactions, Tie1 and Tie2 compete for binding

(C) Ang1 can bind to the integrin proteins with or without the presence of Tie2

(D) & (E) Tie2 - integrin association promote activation of Tie2 to lower concentrations of Ang-1

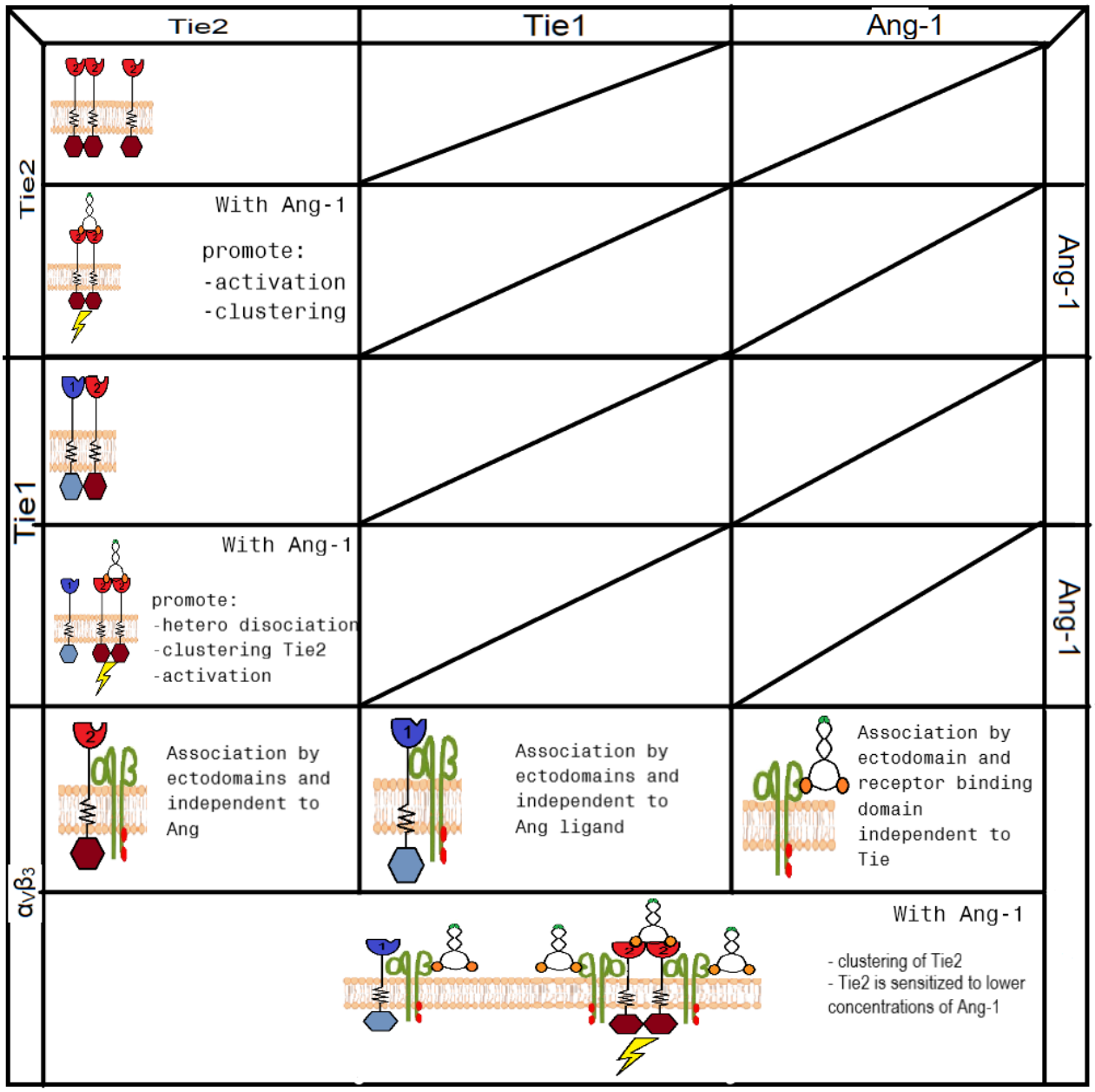


Fig.5. Summary

The Experiment:

Overview:

An antibody against Ang-1 is made by *hybridoma* technology that will be screened by a series of test to ensure that it a) prevents Ang-1 binding to Integrin, and b) still allows Ang-1 binding to Tie-2. Then, activation of Tie-2 will be measured by Western Blotting technique (**Fig.7. and Fig.8**).

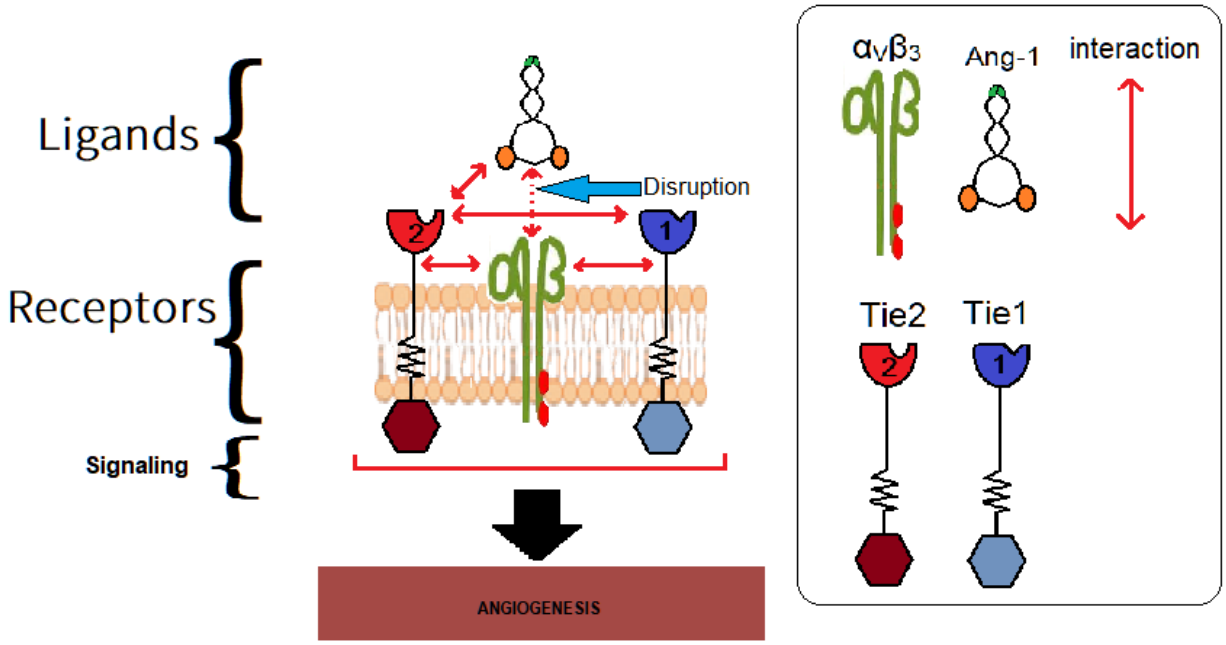


Fig.7. Schematic diagram of experiment

General steps:

Making antibody for Ang-1
Test to the interactions between Tie2 – Ang-1 and $\alpha_V\beta_3$ - Ang-1 with antibodies:

a. Confirming Antibody – Ang1 Binding
b. Association between Tie2 – Ang-1 and αVß3 - Ang-1 with presence of antibodies.

Select a specific antibody that blocks ONLY the association between $\alpha_V\beta_3$ and Ang-1,
Test the influences of selected antibody on Ang-1 activity, and Tie2 activity.

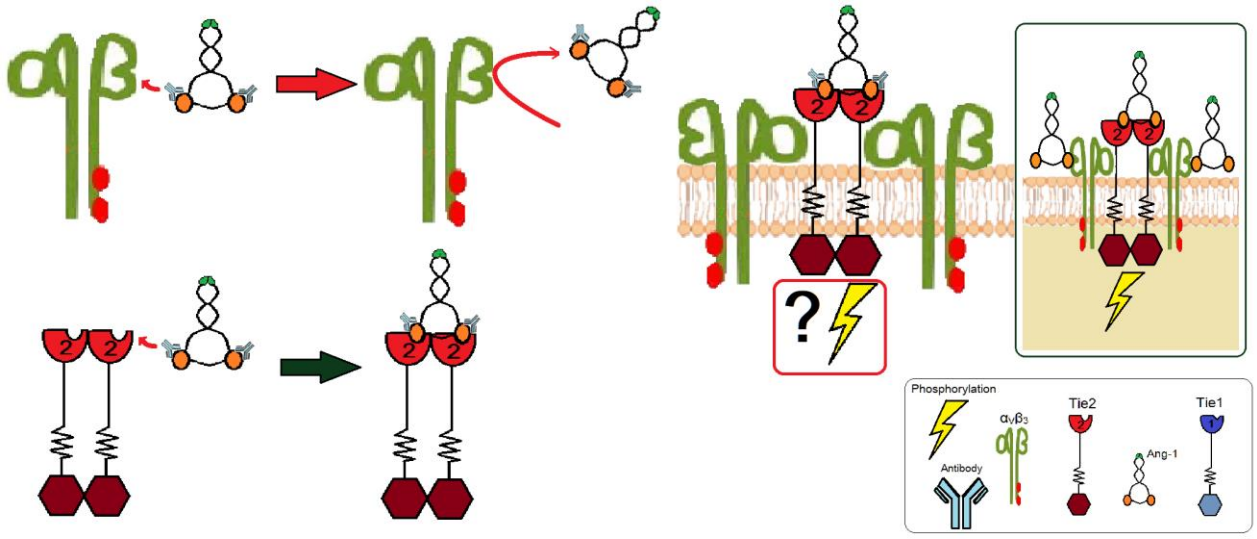


Fig.8. Model of disruption of Angiopoietin-1 and αvβ3 integrin interaction by antibodies

Step I: Making antibodies for Ang-1

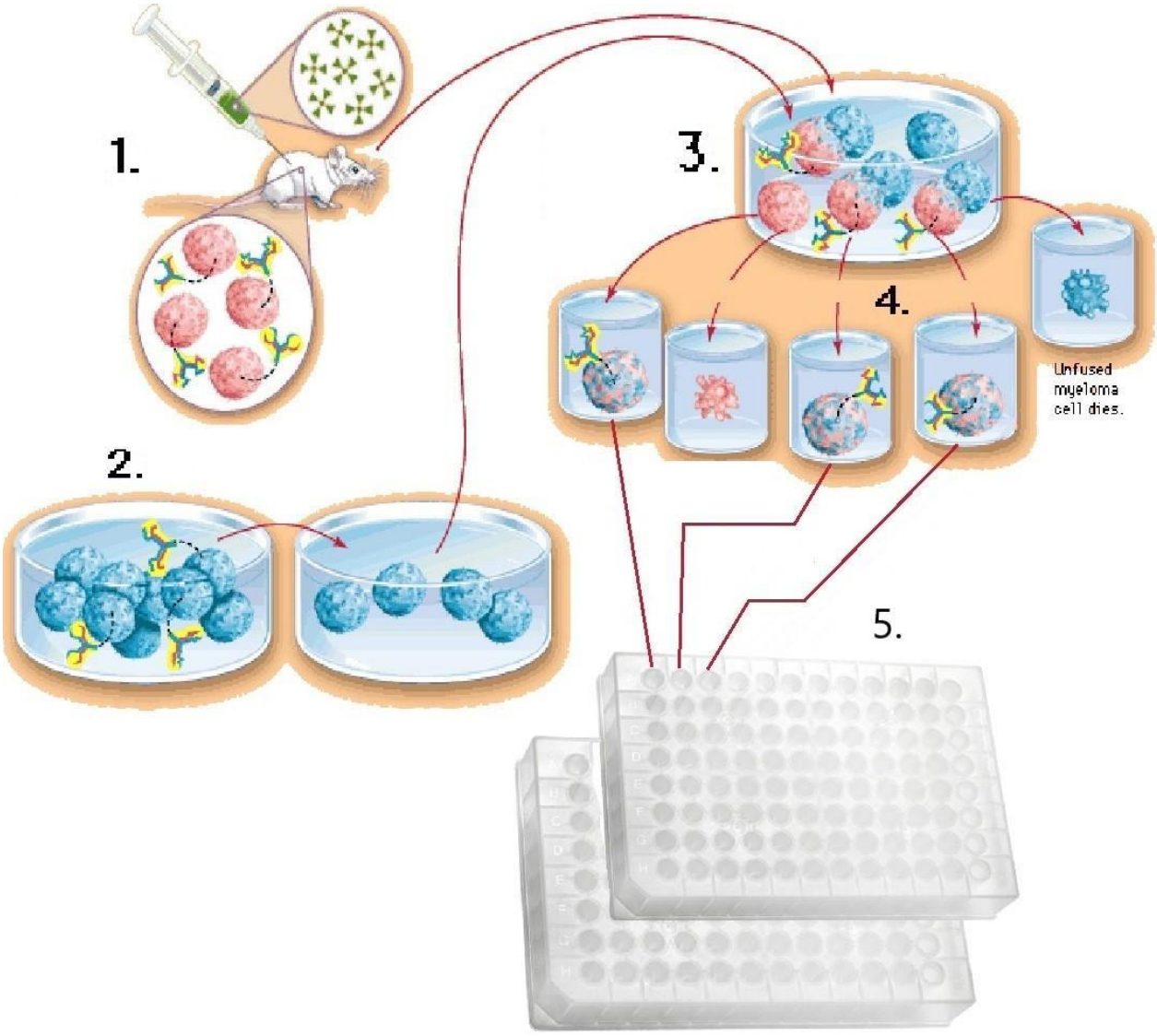


Fig.9. Making Antibodies: general steps redrawn from [28-29].

1.Mouse is injected with Angiopoietin-1 . (Ang-1 purification can be found in [30] method) mouse spleen produces plasma cell that product and secrete antibodies against Ang-1.

2. Select Myeloma cells (cancerous plasma cells) that can't produce antibodies, or purchase them [See more, 4.1 Preparation of Myeloma Cells].[31]

3. Plasma cells from the spleen are isolated and mixed with Myeloma cells to induce cell fusion, resulting in hybridomas

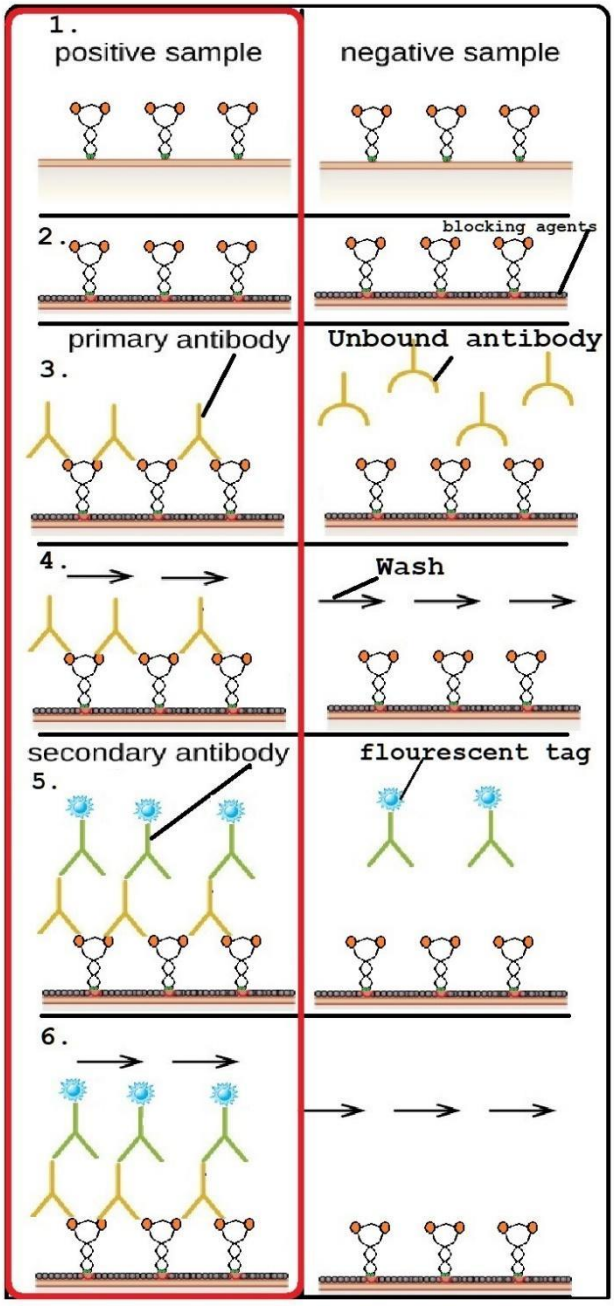
4. Hybridomas are grown in *HAT medium*. Cells that successfully fused (once they are fused successfully, they are alive, producing and secreting antibodies) are selected and unfused cells are discarded. Unfused spleen cells do not replicate and thus selected against, by using HAT

medium, unfused myelomas are selected against, due to an ingredient in the medium block DNA synthesis pathways [See more].[32]

5. Hybridomas that survived are then transferred to 96-well plates with HAT medium one cell in each well. Each well acts as a *monoclonal* culture, each hybridoma in a well inherits the longevity of myeloma cells, replicates, produces and secretes monoclonal antibodies. THESE PLATES WILL SERVE AS THE ORIGINAL TEMPLATES.

6. Making copies of the original templates, the copy's supernatants (which contain secreted antibodies) will be used for the rest of the experiment instead of the originals to conserve and prevent contamination in the original sources.

Step II: Confirming Antibody – Ang1 Binding



This step ensure that the antibodies produced from step I will bind to Ang-1.

Fig.10. Confirming Antibody – Ang1 Binding by Indirect *ELISA*

1. **Coating antigen to microplate:**
ELISA fresh microplates (plates with protein-binding sites on the surface) are set up in the same arrangement as the original templates (96-well plates) from Step I to facilitate uniformity.
Angiopoietin-1 is added to coat the fresh plates [following general protocol: Indirect-ELISA]

2. **Blocking**:
Blocking buffer and 5% non-fat dry milk (available in the ELISA kit) is added to cover the rest of the protein-binding sites on the plates' surfaces; the coverage by blocking agents prevent any additional proteins in the following steps to adhere to the plate surface.
[following general protocol: Indirect-ELISA]

3. **Adding primary antibody**:
The supernatants from each well from the copy in **Step I.6** (each contains monoclonal antibody) are individually pipetted into **Step II.2** Ang-1 coated plates in the following fashion:
Step I.6. Well#1 supernatant to **Step II**. Well #1
Step I.6. Well#2 supernatant to **Step II**. Well #2
And so-forth

4. **Wash**:
Using *Phosphate-buffered saline (PBS)* buffer to wash away antibodies that don't bind well to Ang-1 that anchored on the plates.

2. **Adding secondary antibody**:

Secondary antibody with fluorescent tag (store-bought with green-fluorescent Alexa Fluor® 488 dye molecule: See more) is added to all Step II microplates. The secondary antibody binds to *Fc region* of primary antibody.

[see more]

3. **Wash**:

Using PBS to wash all the wells, unbound secondary antibody, this step may be repeated.

4. **Visualization:**

Excitation with short wavelength blue-green light (more on wavelength can be found in [33]) is used to read the ELISA plates. Wells emitting strong green fluorescent light indicate the binding of primary antibody to Ang-1 (left panel in red), are selected. The copy plates in **Step I.6** will be marked "Binds to Ang-1" accordingly to the selected plates:

For example: if ELISA well #1 emit a strong fluorescent, **Step I.6** well #1 is marked "Binds to Ang-1".

In vitro: Association between Tie2 – Ang-1 and $\alpha_V ß_3$ - Ang-1 in the presence of antibodies.

Step III: Tie2 – Ang-1

This step ensures antibody will allow Ang-1 binding to Tie2.

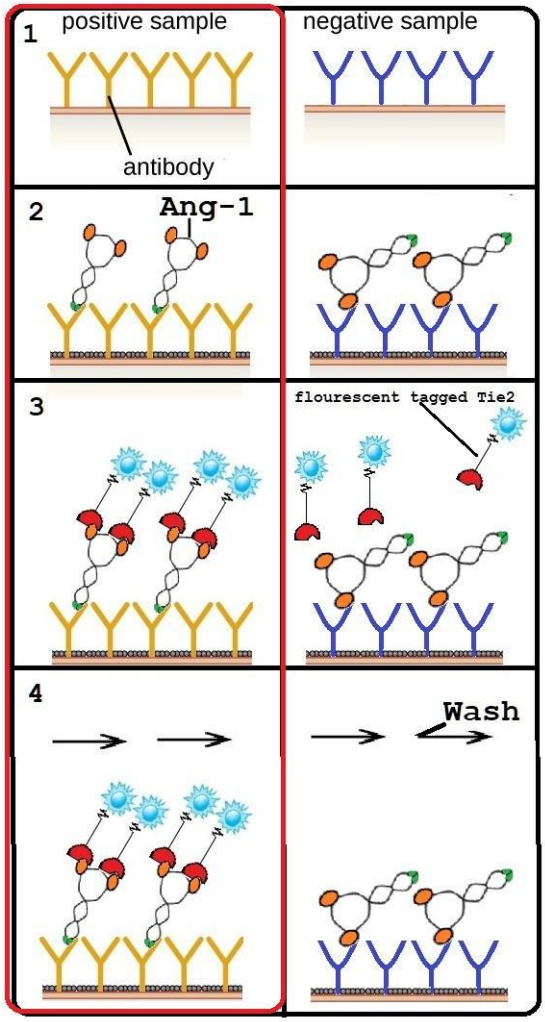


Fig.11. In vitro: Confirming Antibody – Ang1 Binding with Tie2 by modified ELISA

1. **Coating antibody to microplate:**
ELISA fresh microplates (plates with protein-binding sites on the surface) are set up in the same arrangement as the original templates (96-well plates) from Step I to facilitate uniformity.
Wells' supernatants are added to coat the fresh plates.
NOTE: Step III and IV start with the same set up. This step is repeated to prepare for Step IV.1
[following general protocol: Sandwich-ELISA]

2. **Adding of Ang-1:**

Ang-1 is added to all the coated wells in **step III.1**

3. **Adding Ectodomain Fluorescent-tagged Tie2:**

Ectodomain Fluorescent-tagged Tie2 [Purification of Tie2,[34] using Protein Labeling Kits store-bought with green-fluorescent Alexa Fluor® 488 dye molecule] to all the wells in **step III.2**. Left panel depicts antibody that bind Ang-1 in a way that the receptor binding domain of Ang-1 is still available for the binding of Tie2.

4. **Wash**:

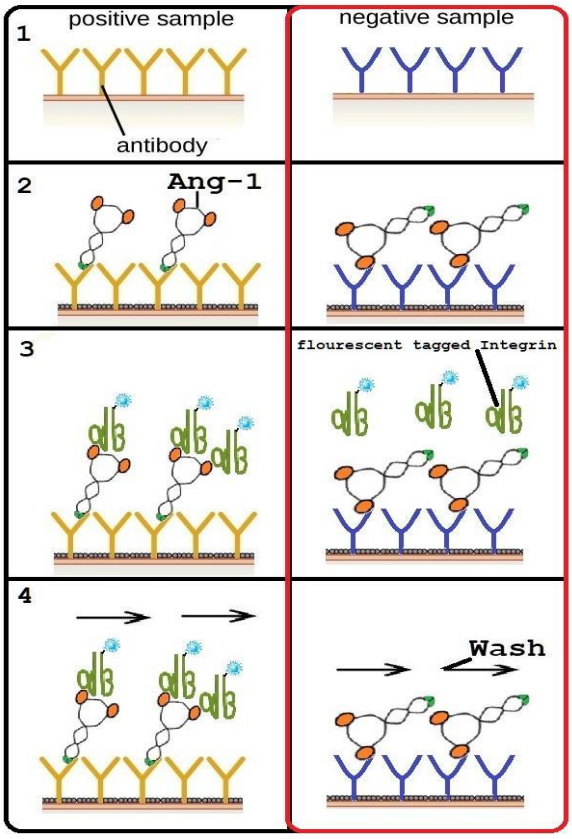
Using PBS to wash all the plates, unbound Tie2 is washed away.

5. **Visualization:**

Excitation with short wavelength blue-green light (more on wavelength can be found in [33]) is used to read the ELISA plates. Plates emitting strong green fluorescent light indicate the binding of Fluorescent-tagged Tie2 to Ang-1 (left panel in red), are selected. The copy plates in **Step I.6** will be marked "Allows Tie-2 binding" accordingly to the selected plates:

For example: if ELISA well #4 emits a strong fluorescence, **Step I.6** well #4 is marked "Allows Tie-2 binding".

Step IV: $\alpha_V\beta_3$ – Ang-1



This step ensures antibody will prevent Ang-1 binding to $\alpha_V\beta_3$.

Fig.12. In vitro: Confirming Antibody – Ang1 Non-Binding with αVß3 integrin by modified ELISA

1. **Coating antibody to microplate:**

Copy plates are prepared in step III.1

2. **Adding of Ang-1:**

Ang-1 is added to all the coated wells in **step IV.1**

3. **Adding Ectodomain Fluorescent-tagged Integrin $\alpha_V\beta_3$:**

Ectodomain Fluorescent-tagged Integrin $\alpha_V\beta_3$ [purification of Integrin,[1] using Protein Labeling Kits store-bought with green-fluorescent Alexa Fluor® 488 dye molecule] to all the wells in **step IV.2**. Left panel depict antibody that bind Ang-1 in a way that the receptor binding domain of Ang-1 is still available for the binding of Integrin $\alpha_V\beta_3$. However, in Step IV, the desirable result is depicted in the right panel: antibody that bind Ang-1 in a way that the receptor binding domain of Ang-1 is NOT available for the binding of Integrin $\alpha_V\beta_3$

4. **Wash**:

Using PBS to wash all the plates, unbound Integrin is washed away.

5. **Visualization:**

Excitation with short wavelength blue-green light (more on wavelength can be found in [33]) is used to read the ELISA plates. Plates emitting strong green fluorescent light indicate the binding of

Fluorescent-tagged Integrin to Ang-1 (left panel), are skipped. Plates that emit weak to no fluorescent light, which indicate the disruption of Fluorescent-tagged Integrin to Ang-1 (right panel), are selected. The copy plates in **Step I.6** will be marked “prevents integrin binding” accordingly to the selected plates:

> For example: if ELISA well #4 does not emit fluorescent, **Step I.6** well #4 is marked “prevents integrin binding”.

Step III-IV: results comparison

Wells that have the desirable antibody will have all three following labels:

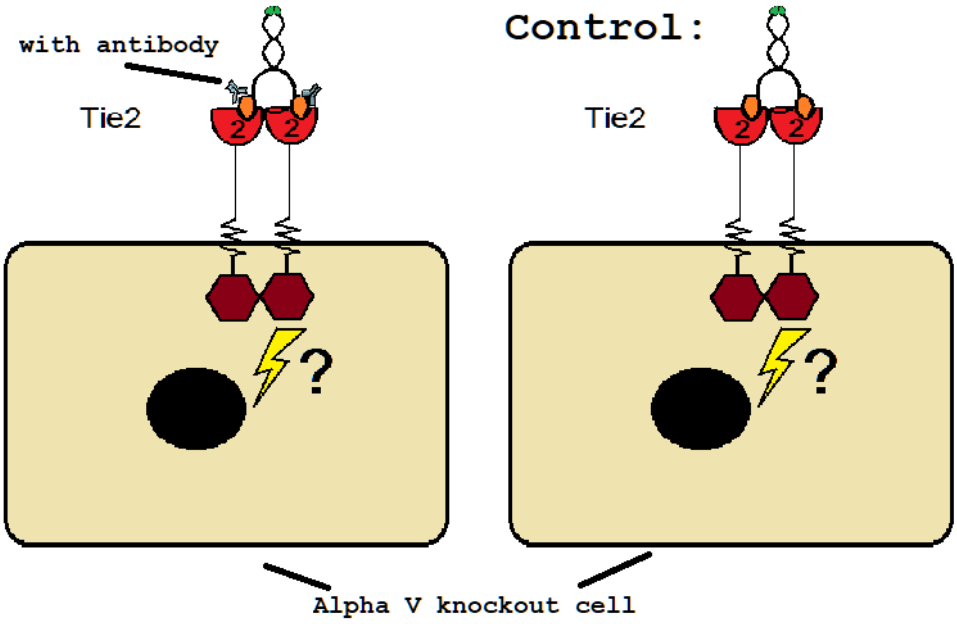
“Binds to Ang-1”, “Allows Tie-2 binding”, and “Prevents integrin binding”.

The antibodies from those wells can be proceeded to **step V** or **step VII**

(Optional) In vivo: Association between Tie2 – Ang-1 and $\alpha_V\beta_3$ - Ang-1 with presence of antibodies.

Step V: Tie2 – Ang-1

Goal: to ensure the activation of Tie2 by Ang-1 is not impaired by the antibody.



Using cell without $\alpha_V\beta_3$ expression to test Tie2 activation without integrin’s interference. Only knockout α_V is enough to bringdown $\alpha_V\beta_3$ complex

Knockout α_V can be ordered through a couple companies [similar products: See more]

Fig.13. In vivo: Confirming Antibody – Ang1

Binding with Tie2 in α_V knock off cells

In α_V knock out cells Subject: Ang-1 is added with the presence of one of the antibodies selected from **steps III-IV.**

Control: Ang-1 is added without the presence of antibody.

Phosphorylation of Tie2 in both samples are measured using Western Blotting with Anti-phosphotyrosine immunoprecipitates (described in step VII). Antibodies that give the same reading with control, meaning they don't affect Tie2 phosphorylation activity, are selected.

Step VI: $\alpha_V\beta_3$ – Ang-1

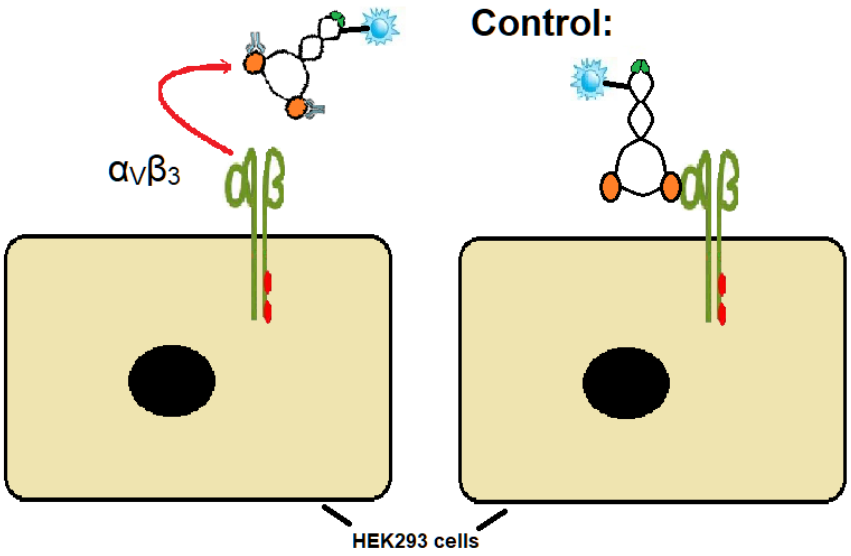


Fig.14. In vitro: Confirming Antibody – Ang1 Binding with αVß3 integrin in HEK293 cell line (lack of tie receptors)

In HEK293 cells:

Subject: fluorescent-tagged Ang-1 [using Protein Labeling Kits store-bought with green-fluorescent Alexa Fluor® 488 dye molecule] is added with the presence of antibody selected from **steps III-IV.**

Control: fluorescent-tagged Ang-1 is added without the presence of antibody

Washing the samples and using microscope to observe the fluorescent on the cell surface, antibodies that do not attach to integrin are selected (no fluorescent is observed in the cell).

Step V-VI: results comparison

Antibodies that were selected from both Step V and Step VI will qualify:

1. Bind to Ang-1 but don't affect activation of Tie2
2. Unable to bind to Integrin $\alpha_V\beta_3$

And they are ready to go to **step VII**

Step VII: Measure Tie2 activity in presence of Ang-1 antibody and Integrin $\alpha_V\beta_3$

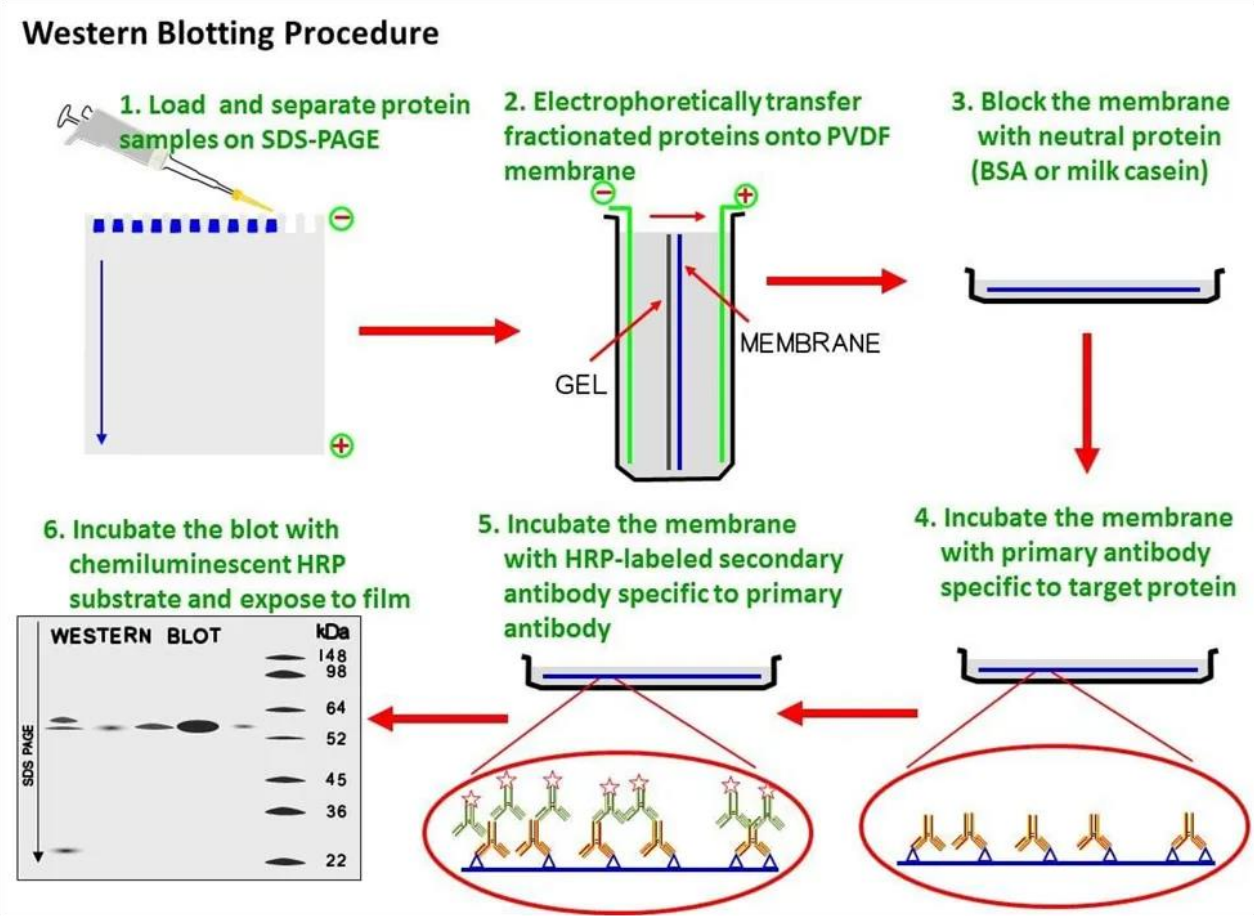


Fig.15. Western Blotting with Anti-phosphotyrosine immunoprecipitates as primary antibody [35]

1. **Protein samples prepared and separation:**

Each well column are loaded with the following compositions:
Reference columns:

Column 1: Tie2
Column 2: $\alpha_V\beta_3$
Column 3: $\alpha_V\beta_3$ + Tie2
Column 4: Ang-1
Column 5: Ang-1 + $\alpha_V\beta_3$
Column 6: Ang-1 + Tie2
Column 7: $\alpha_V\beta_3$ +Tie2 with Ang-1

Test column:

Column 8: Integrin+Tie2 with Ang-1 and the antibody selected from **step V-VI** (or **step III-IV**)

Using gel electrophoresis to separate protein by weight. Charged protein transported through the gel by an electric field.

2. **Transfer to membrane:**

Once separated, all the proteins are transferred to a membrane (by another electric field) which supports protein detection

3. **Blocking:**

Adding blocking agent to the membrane to prevent the antibody from sticking to the membrane

4. **Addition of primary antibody:**

Anti-phosphotyrosine antibody (store-bought: See more) is added as the primary antibody. Anti-phosphotyrosine antibody recognizes tyrosine phosphorylated proteins (Tie2 autophosphorylation in this experiment .[36] Then the membrane is washed off excess unbound primary antibodies by PBS buffer.

5. **Addition of secondary antibody:**

Secondary antibody tagged with fluorescent tag (store-bought with green-fluorescent Alexa Fluor® 488 dye molecule: See more) is added, then washed off any excess. This secondary antibody is specific to the primary antibody (like binding to the Fc region of the primary antibody)

6. **Visualization:**

Expose the membrane to short wavelength blue-green light to get the reading

Discussion:

The level of autophosphorylation of Tie 2 can be observed in column 6, 7, and 8. Our interest is the activity level in the presence of the chosen antibody (column 8), while column 6 and 7 serve as control.

Predictions:

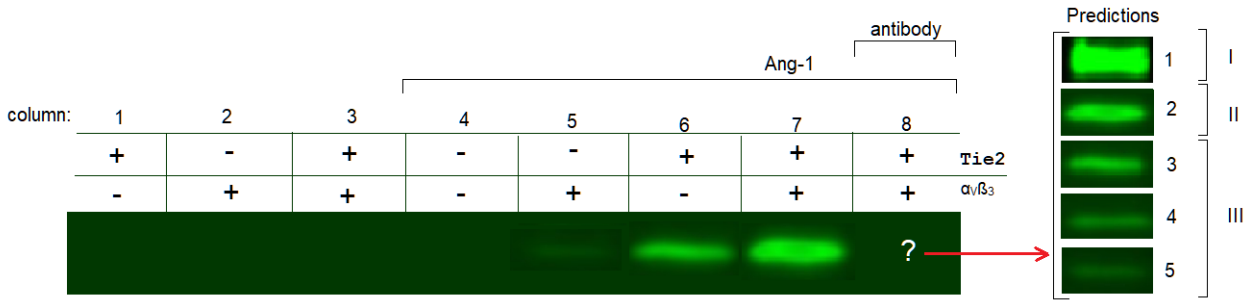


Fig.16. Predictions of Tie2 activities with/without presence of antibody that disrupts Integrin αVß3 -Ang1 association by Western blotting.

Prediction I: Higher level of Tie2 autophosphorylation

- Speculation:
  - Could be a result from more Ang-1 molecules available, since they don't have to bind to Integrin, and the association of Ang-1 to Integrin doesn't affect Tie2 clustering, and/or Tie2 more sensitive to Ang-1; i.e. Integrin by itself (without

binding to Ang-1) can facilitate Tie2 clustering and/or Tie2 higher sensitivity to Ang-1.
    - Disrupting the Ang-1/ $\alpha_V\beta_3$ can't be used as an inhibitory method to Tie2 activation.

Prediction II: Same level of Tie2 autophosphorylation
- Speculation:
    - Could be a result from more Ang-1 molecules available, since they don't have to bind to Integrin. However, the association of Ang-1 to Integrin slightly lower Tie2 clustering, and/or Tie2 more sensitive to Ang-1; i.e. Integrin by itself (without binding to Ang-1) can facilitate Tie2 clustering and/or Tie2 higher sensitivity to Ang-1 but not effective, results in the same level of Tie2 autophosphorylation.
    - Disrupting the Ang-1/ $\alpha_V\beta_3$ can't be used as an inhibitory method to Tie2 activation.

Prediction III: Lower level of Tie2 autophosphorylation
- Speculation:
    - Despite of higher level of Ang-1 molecules available (blocked from binding to Integrin), it could be that the association of Ang-1 to Integrin significantly lower Tie2 clustering, and/or Tie2 more sensitive to Ang-1; i.e. Integrin by itself (without binding to Ang-1) can't facilitate Tie2 clustering and/or Tie2 higher sensitivity to Ang-1, and/or affect the ability to disrupt heterodimer tie2-tie1, results in the lower level of Tie2 autophosphorylation.
    - Disrupting the Ang-1/ $\alpha_V\beta_3$ can be used as an inhibitory method to Tie2 activation.

Final words:

The experiment only looks at the level of autophosphorylation of Tie2 in association with $\alpha_V\beta_3$ by its ligand Ang-1 (in which $\alpha_V\beta_3$ does not bind to Ang-1). The effects of Ang-1 on $\alpha_V\beta_3$ and how that affects Tie-2 activation is not in the scope of this experiment.

If by blocking Ang-1 binding to $\alpha_V\beta_3$ lowers Tie2 activation, it could become a new drug target. Thus, it can be coupled with other Tie2 inhibitors to create compounded medication.

Glossary:

$\alpha_x\beta_y$: integrin proteins, composed of α and ß peptide chains.

Agonist: a chemical that binds to a receptor and activates the receptor to produce a biological response.

Prefix: **Angio**-

relating to (blood) vessels.

Angiogenesis: Forming of blood vessels.

Angiopoietins: ligands of Tie receptors

Antibody: a blood protein produced in response to and counteracting a specific antigen

Auto-phosphorylation: is a biochemical process in which a phosphate group is added to a protein kinase by the action of the protein kinase itself

Cardiomyocyte: cells responsible for generating contractile force in the intact heart

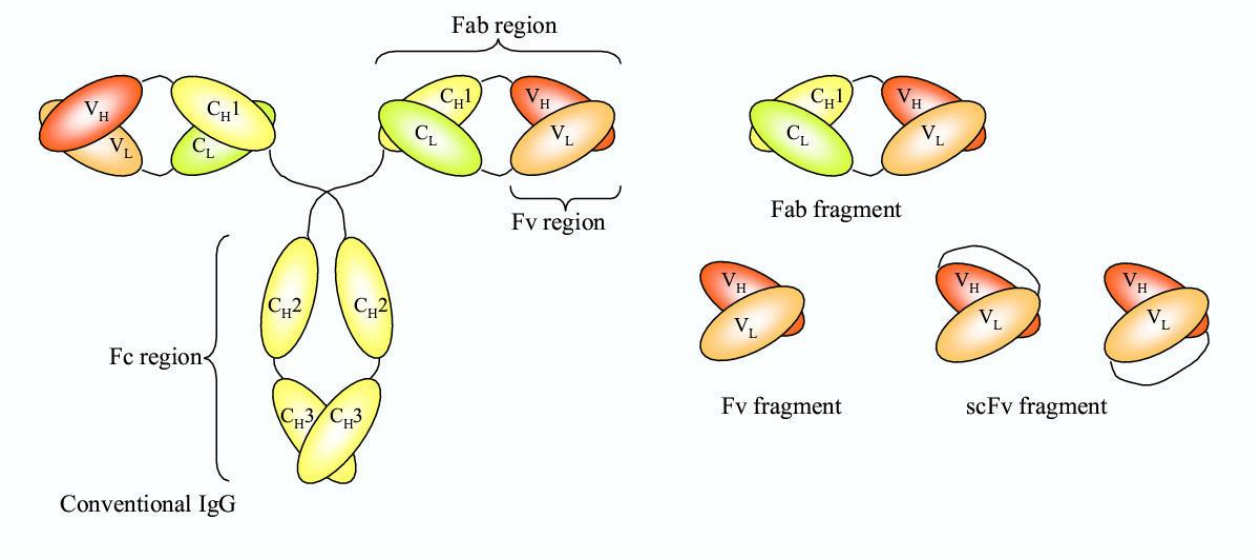
Ectodomain*:* is the part of a membrane protein that extends into the space outside the cell

ELISA: enzyme-linked immunosorbent assay, a technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. See more

Endogenous: within an organism

Endothelial*:* cells that line the interior surface of vessels

Fc region of antibody:



See more.

HAT medium: is a selection medium for mammalian cell culture. See more

Heterodimer dissociation: in this context, it means the dissociation of Tie1-Tie2 complex

Hybridoma: B cell, produces antibodies fused with immortal B cell cancer cells, a myeloma to produce a hybrid cell line called a hybridoma, which has both the antibody-producing ability of the B-cell and the longevity and reproductivity of the myeloma.

Monoclonal: clone that is derived asexually from a single individual or cell.

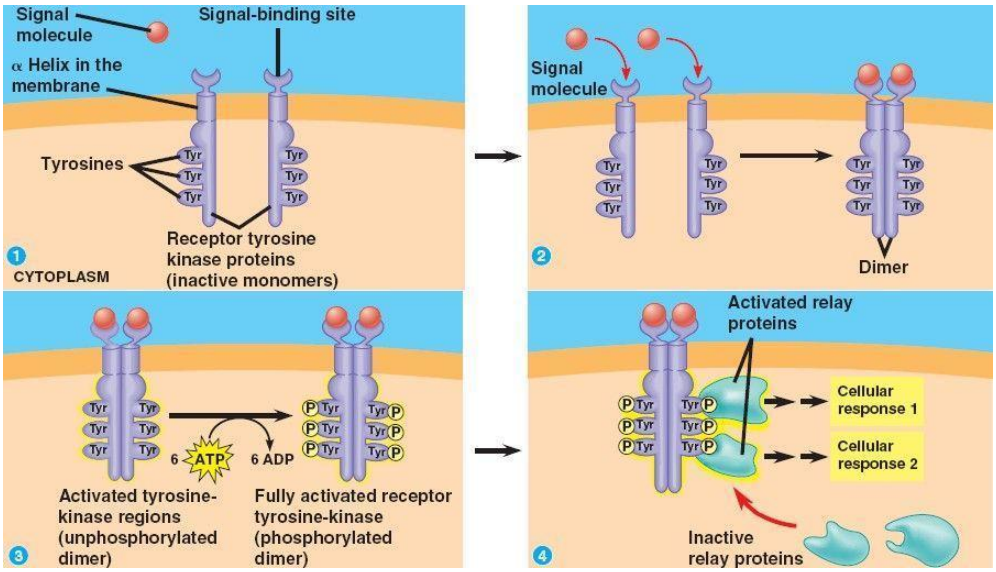
Phosphate-buffered saline (PSS): is a buffer **solution** commonly used in biological research. It is a water-based salt **solution** containing disodium hydrogen phosphate, sodium chloride and, in some formulations, potassium chloride and potassium dihydrogen phosphate. See more

Pulmonary edema: excess fluid swelling in the lungs.

Receptor Tyrosine Kinases (RTKs): "Protein tyrosine kinases are enzymes that are capable of adding phosphate groups to specific tyrosines on target proteins. A receptor tyrosine kinase

(RTK) is a tyrosine kinase located at the cellular membrane and is activated by binding of a ligand via its extracellular domain."[37]

Tie: primary receptor for angiopoietin ligands, a type of endothelial cells protein tyrosine kinase play a crucial role for angiogenesis and vascular maintenance



Utero: in the womb

References:

1. https://www.cancer.gov/about-cancer/understanding/statistics
2. https://www.who.int/news-room/fact-sheets/detail/cancer
3. Loizzi, V., Vecchio, V.D., Gargano, G., Liso, M.D., Kardashi, A., Naglieri, E., Resta, L., Cicinelli, E., & Cormio, G. (2017). Biological Pathways Involved in Tumor Angiogenesis and Bevacizumab Based Anti-Angiogenic Therapy with Special References to Ovarian Cancer. International journal of molecular sciences.
4. Paweletz, N., & Boxberger, H. J. (1994). Defined tumor cell-host interactions are necessary for malignant growth. *Critical Reviews in Oncogenesis*, *5*(1), 69–105. https://doi.org/10.1615/critrevoncog.v5.i1.40
5. Khan, K., Cunningham, D., & Chau, I. (2017). Targeting Angiogenic Pathways in Colorectal Cancer: Complexities, Challenges and Future Directions. *Current Drug Targets*, *18*(1), 56–71. https://doi.org/10.2174/1389450116666150325231555
6. Dalton, Annamarie & Shlamkovich, Tomer & Papo, Niv & Barton, William. (2016). Constitutive Association of Tie1 and Tie2 with Endothelial Integrins is Functionally Modulated by Angiopoietin-1 and Fibronectin. PLOS ONE. 11. 10.1371/journal.pone.0163732.

7. Lin P, Polverini P, Dewhirst M, Shan S, Rao PS, Peters K. Inhibition of tumor angiogenesis using a soluble receptor establishes a role for Tie2 in pathologic vascular growth. The Journal of clinical investigation 1997;100:2072–2078. [PubMed: 9329972]
8. Bogdanovic E, Nguyen VP, Dumont DJ. Activation of Tie2 by angiopoietin-1 and angiopoietin-2 results in their release and receptor internalization. J Cell Sci. 2006 Sep 1;119(Pt 17):3551-60. doi: 10.1242/jcs.03077. Epub 2006 Aug 8. PubMed PMID: 16895971.
9. Davis, S., Aldrich, T. H., Jones, P. F., Acheson, A., Compton, D. L., Jain, V., Ryan, T. E., Bruno, J., Radziejewski, C., Maisonpierre, P. C. et al. (1996). Isolation of angiopoietin-1, a ligand for the Tie2 receptor, by secretion-trap expression cloning. Cell 87, 1161-1169
10. Witzenbichler, B., Maisonpierre, P. C., Jones, P., Yancopoulos, G. D. and Isner, J. M. (1998). Chemotactic properties of angiopoietin-1 and –2, ligands for the endothelialspecific tyrosine kinase Tie2. J. Biol. Chem. 273, 18514-18521.
11. Jones, N., Master, Z., Jones, J., Bouchard, D., Gunji, Y., Sasaki, H., Daly, R., Alitalo, K. and Dumont, D. J. (1999). Identification of Tek/Tie2 binding partners. J. Biol. Chem. 274, 30896-30905.
12. Hayes, A. J., Huang, W. Q., Mallah, J., Yang, D., Lippman, M. E. and Li, L. Y. (1999). Angiopoietin-1 and its receptor Tie-2 participate in the regulation of capillary-like tube formation and survival of endothelial cell. Microvasc. Res. 58, 224-237
13. Koblizek, T. I., Weiss, C., Yancopoulos, G. D., Deutsch, U. and Risau, W. (1998). Angiopoietin-1 induces sprouting angiogenesis in vitro. Curr. Biol. 8, 529-532.
14. Maisonpierre, P. C., Suri, C., Jones, P. F., Bartunkova, S., Wiegand, S. J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T. H., Papadopoulos, N. et al. (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277, 55-60.
15. Seegar, Tom & Eller, Becca & Tzvetkova-Robev, Dorothea & Kolev, Momchil & Henderson, Scott & Nikolov, Dimitar & Barton, William. (2010). Tie1-Tie2 Interactions Mediate Functional Differences between Angiopoietin Ligands. Molecular cell. 37. 643-55. 10.1016/j.molcel.2010.02.007.
16. Fioramonti, M., Fausti, V., Pantano, F. *et al.* Cabozantinib Affects Osteosarcoma Growth Through A Direct Effect On Tumor Cells and Modifications In Bone Microenvironment. *Sci Rep* **8,** 4177 (2018) doi:10.1038/s41598-018-22469-5
17. Garcia-Manero G, Khoury HJ, Jabbour E, et al. A phase I study of oral ARRY-614, a p38 MAPK/Tie2 dual inhibitor, in patients with low or intermediate-1 risk myelodysplastic syndromes. Clin Cancer Res. 2015;21(5):985–994. doi:10.1158/1078-0432.CCR-14-1765

18. Liontos M, Lykka M, Dimopoulos MA, Bamias A. Profile of trebananib (AMG386) and its potential in the treatment of ovarian cancer. Onco Targets Ther. 2014;7:1837–1845. Published 2014 Oct 4. doi:10.2147/OTT.S65522

19. Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., … Walter, P. (2013). Essential Cell Biology, 4th Edition [Hardcover].

20. Carlson TR, Hu H, Braren R, Kim YH, Wang RA. Cell-autonomous requirement for beta1 integrin in endothelial cell adhesion, migration and survival during angiogenesis in mice. Development. 2008; 135: 2193–2202. doi: 10.1242/dev.016378 PMID: 18480158

21. Tanjore H, Zeisberg EM, Gerami-Naini B, Kalluri R. Beta1 integrin expression on endothelial cells is required for angiogenesis but not for vasculogenesis. Dev. Dynam. 2008; 237: 75–82. doi: 10.1002/ dvdy.21385 PMID: 18058911

22. Lee HS, Oh SJ, Lee KH, Lee YS, Ko E, Kim KE, et al. Gln-362 of angiopoietin-2 mediates migration of tumor and endothelial cells through association with alpha5beta1 integrin. J. Biol. Chem. 2014; 289: 31330–31340. doi: 10.1074/jbc.M114.572594 PMID: 25237190

23. Chen X, Fu W, Tung CE, Ward NL. Angiopoietin-1 induces neurite outgrowth of pc12 cells in a tie2-independent, beta1-integrin-dependent manner. Neurosci. Res. 2009; 64: 348±354. doi: 10.1016/j.neures.2009.04.007 PMID: 19379779

24. Dallabrida SM, Ismail NS, Pravda EA, Parodi EM, Dickie R, Durand EM, et al. Integrin binding angiopoietin-1 monomers reduce cardiac hypertrophy. FASEB J. 2008; 22: 3010±3023. doi: 10.1096/fj.07-100966 PMID: 18502941

25. Imanishi Y, Hu B, Jarzynka MJ, Guo P, Elishaev E, Bar-Joseph I, et al. Angiopoietin-2 stimulates breast cancer metastasis through the alpha5beta1 integrin mediated pathway. Cancer Res. 2007; 67: 4254±4263. doi: 10.1158/0008-5472.CAN-06-4100 PMID: 17483337

26. Taherian A, Li X, Liu Y, Haas TA. Differences in integrin expression and signaling within human breast cancer cells. BMC Cancer. 2011; 11: 293. doi: 10.1186/1471-2407-11-293 PMID: 21752268

27. Lee EH, Woo JS, Hwang JH, Park JH & Cho CH. Angiopoietin 1 enhances the proliferation and differentiation of skeletal myoblasts. J. Cell. Phys. 2013; 228: 1038±1044. doi: 10.1002/jcp.24251 PMID: 23041942

28. https://www.britannica.com/science/monoclonal-antibody

29. https://www.creative-diagnostics.com/Custom-Antibody-Purification.htm

30. Barton, W. A., Tzvetkova, D., & Nikolov, D. B. (2005). Structure of the angiopoietin-2 receptor binding domain and identification of surfaces involved in Tie2 recognition. *Structure*, Vol. 13, pp. 825–832. https://doi.org/10.1016/j.str.2005.03.009

31. Technologies Inc, S. (n.d.). *ClonaCell™-HY: A Complete Workflow for Hybridoma Generation*.

32. https://www.moleculardevices.com/en/assets/tutorials-videos/reagents/hybridoma-selection-using-hat-medium#gref
33. https://www.thermofisher.com/order/spectra-viewer?SID=srch-svtool&UID=11001ph8
34. Barton, William & Tzvetkova-Robev, Dorothea & Miranda, Edward & Kolev, Momchil & Rajashankar, Kanagalaghatta & Himanen, Juha & Nikolov, Dimitar. (2006). Crystal structures of the Tie2 receptor ectodomain and the angiopoietin-2-Tie2 complex. Nature structural & molecular biology. 13. 524-32. 10.1038/nsmb1101.
35. https://microbeonline.com/western-blot-technique-principle-procedures-advantages-and-disadvantages/
36. https://www.cytoskeleton.com/pdf-storage/datasheets/apy03-s.pdf
37. Department of Neurosurgery, Division of Neuroscience, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa, 920-8640, Japan. mnakada@med.kanazawa-u.ac.jp