Disruption of Angiopoietin-1 and αvβ3 Integrin Interaction in Tie1, Tie2, Ang-1 and αvβ3 Integrin complexes

***Color: Red- Jeff’s comments***

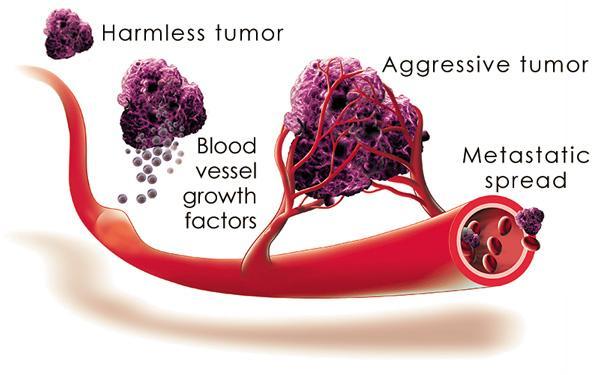
***Blue- Kyle’s comments***

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

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

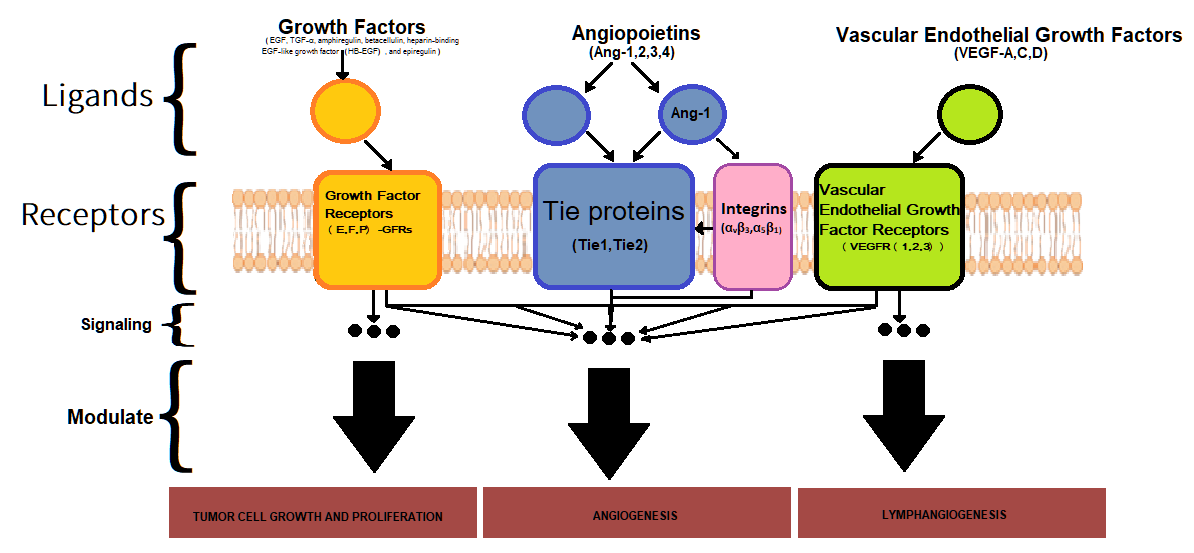
# Introduction:

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 (34,35). 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 35.



**Fig.1. Angiogenesis in tumor cells**

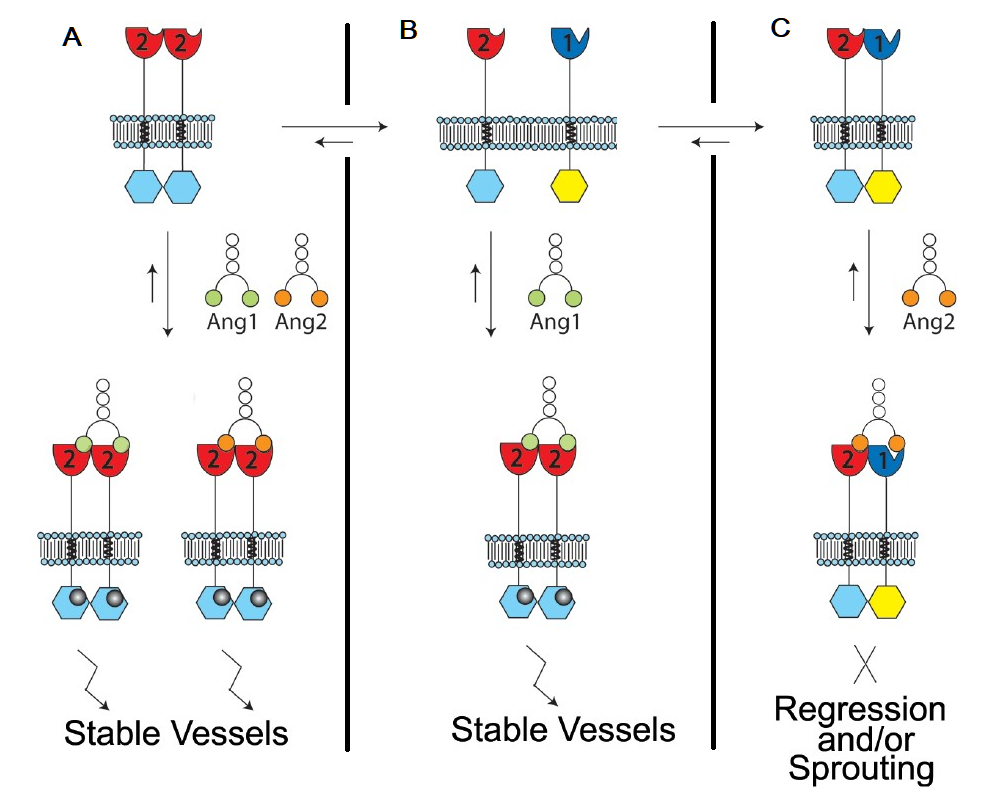
Cancer is a disease when cells in one’s own body has gone wrong. In the early development of cancer, a clump of cancer cells cannot grow larger than a certain size by diffusion. Absorption of nutrients from the environment and waste removing through their surface area are no longer sufficient. A different 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 36. 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 37, with added information from (3,39).

*Tie* receptors and their ligands *Angiopoietins* play a crucial role in regulating tumor-induced angiogenesis (**Fig.2**) 32. **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 vessels maintenance 23. **Angiopoietin-1** (out of 4) Tie2 protein ligands (Ang1-4), has been recognized as the primary activating ligand for Tie2, is a constitutive receptor *agonist* (23,24). 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 (27,28), tube formation 30, sprouting 29 and cell survival 28. **Tie1**, is a transmembrane protein and biological homologous to Tie2, however does not directly interact with any angiopoietins 25. Studies in mice shows that subjects that have a lack of Tie1 likely suffer *pulmonary edema*, vessel integrity defects, localized hemorrhaging, and die *in utero*. Tie1 is speculated to play a role in vessel integrity. However, exact role of Tie1 in angiogenesis is still unknown.

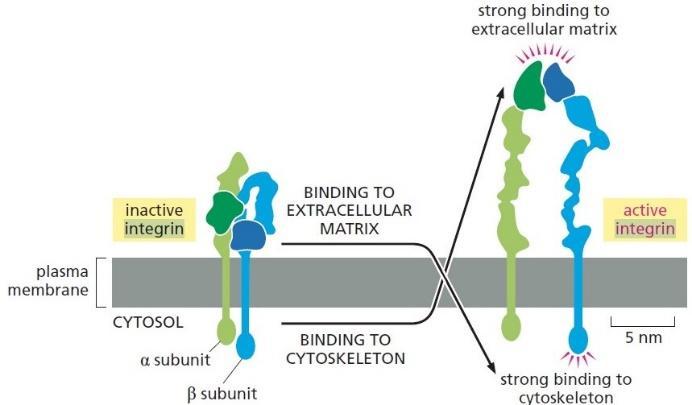
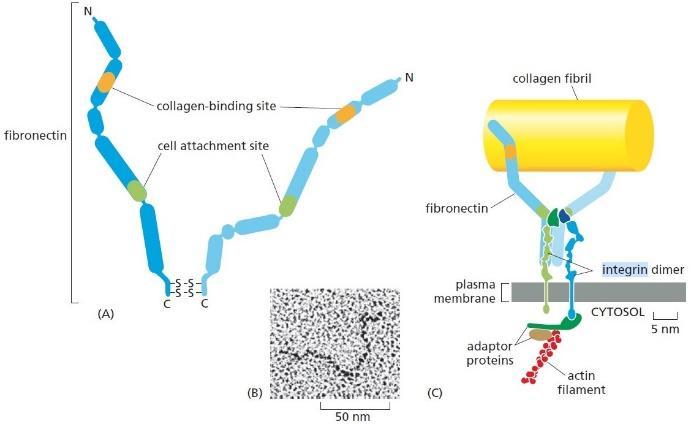
Studies have shown that there is an association between Tie1 and Tie2 on the cell surface, prior to ligand recognition 4. In the presence of Tie1, Tie2 basal activation is drastically decreased (~50% compared to no Tie1) 4. Ang1 and Ang2 exhibit different roles when interacting with the Tie1/Tie2 complex. **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, andsignaling initiation(**Fig.3.B**) *4.*



**Fig.3. Model for Angiopoietin-mediated Tie2 signaling4**

Ang-Tie system certainly is an important drug target to treat tumor. Drugs that are broad-spectrum inhibitors of RTKs (including Tie2) (40,42): Cabozantinib, Lenalidomide, Regorafenib… Inhibiting Angiopoietin 1 & 2 interaction with their receptor Tie2 41: 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 are freely but mutually exclusive interact with the **Integrins** *α5β1* and ***αVβ3*** through their ectodomains (i.e. they are competing for binding with integrin (**Fig.5.row5.column 1 and 2**) 3. The Integrins are membrane protein, that link actin inside the cell to the matrix protein outside of the cell 52.

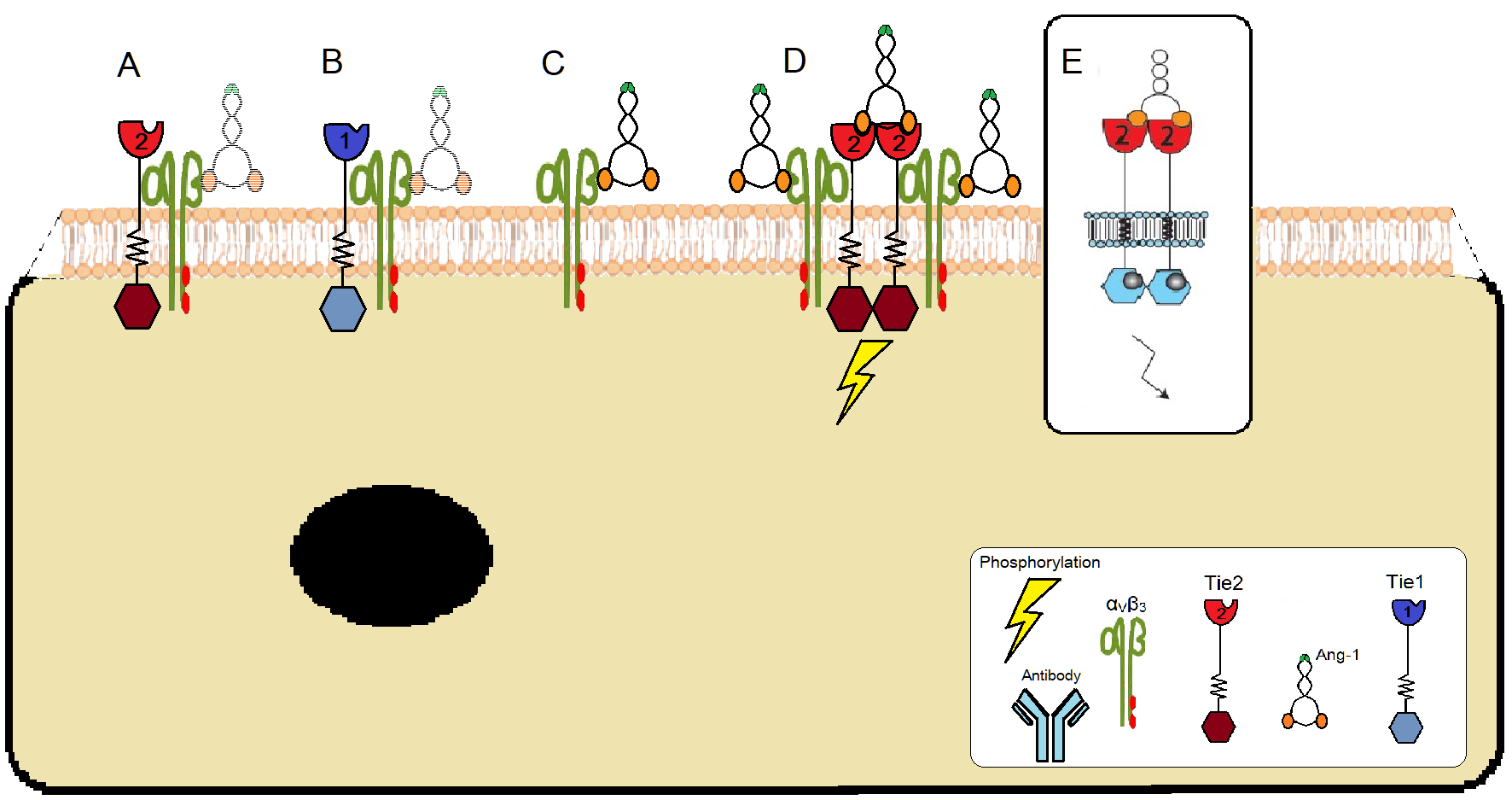


**Fig.3.1. Integrins functions and different conformations**

Integrin-Tie recognition may help split up the Tie1/Tie2 complex 3. 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 α5β1 and αVβ33. Association of 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) is shown to be also capable to independently bind to integrin proteins (**Fig.5.row 5.colum 3**)regardless of Tie2 presence 3. Association with Integrin raise Tie2 sensitivity to of Ang13 (**Fig.5.row 6**).

Studies have also suggested a function for angiopoietin ligands that are dependent on integrin but independent of Tie2(17-19). Angiopoietins induced effects also have been described in *cardiomyocytes*, breast cancer cells, and neurons that Tie2 is not expressed (43-47).

This proposal explores the potential of disrupting Integrin (αVβ3) and Ang-1 association in altering and potentially (speculatively) lowering Tie2 autophosphorylation.



**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 αVß3

**(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

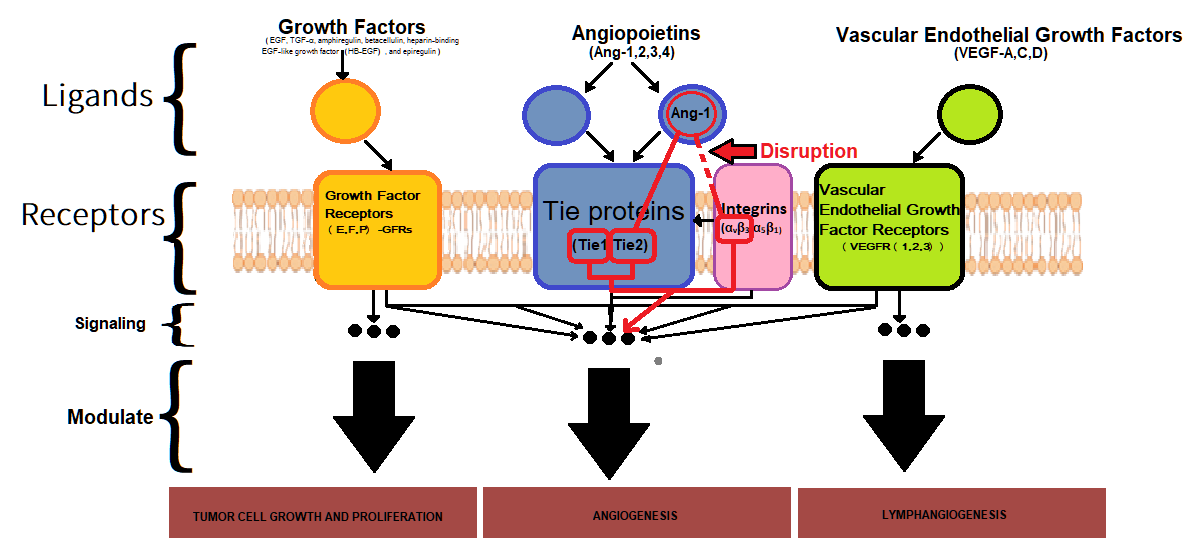
**(D) & (E)** Tie2 - integrin association promote activation of Tie2 to lower concentrations of Ang-1

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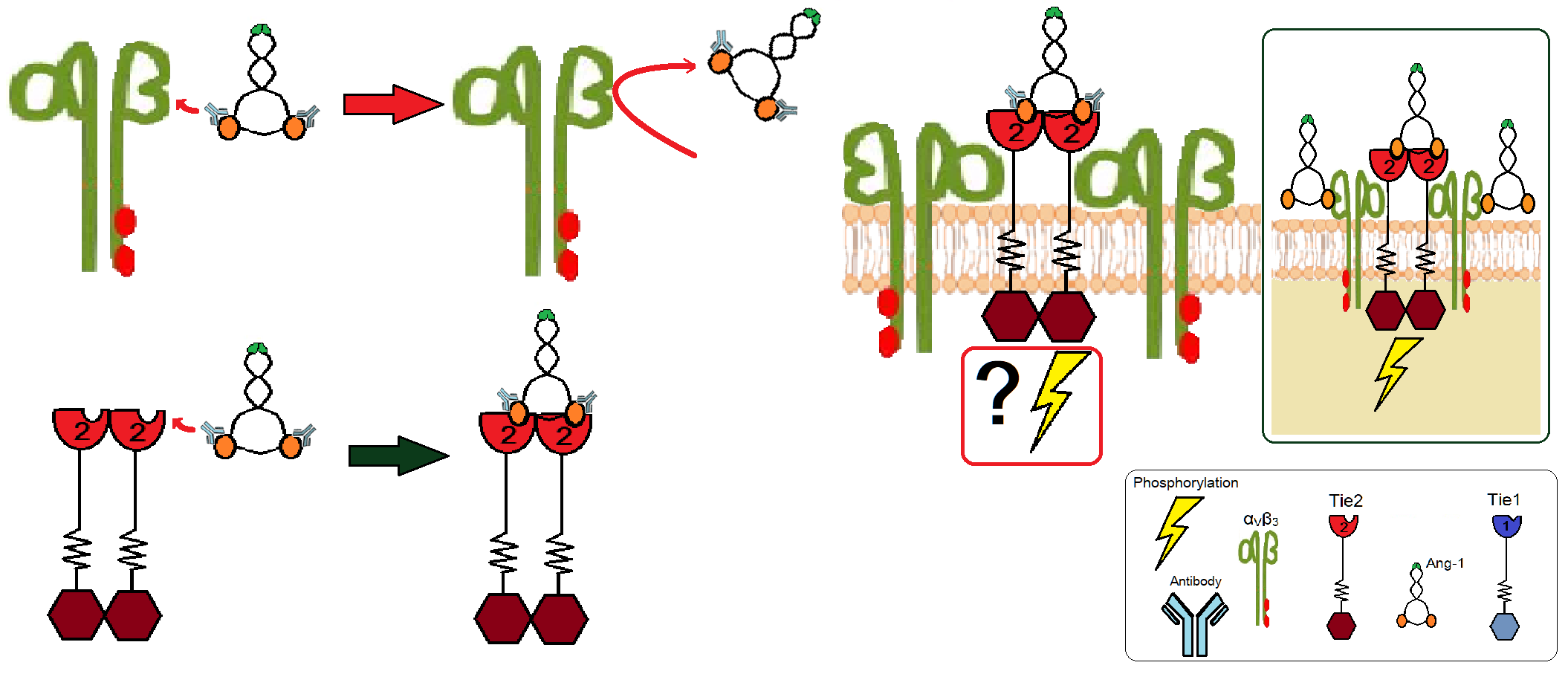
**Fig.5. Summary**

# The Experiment:

## Overview:

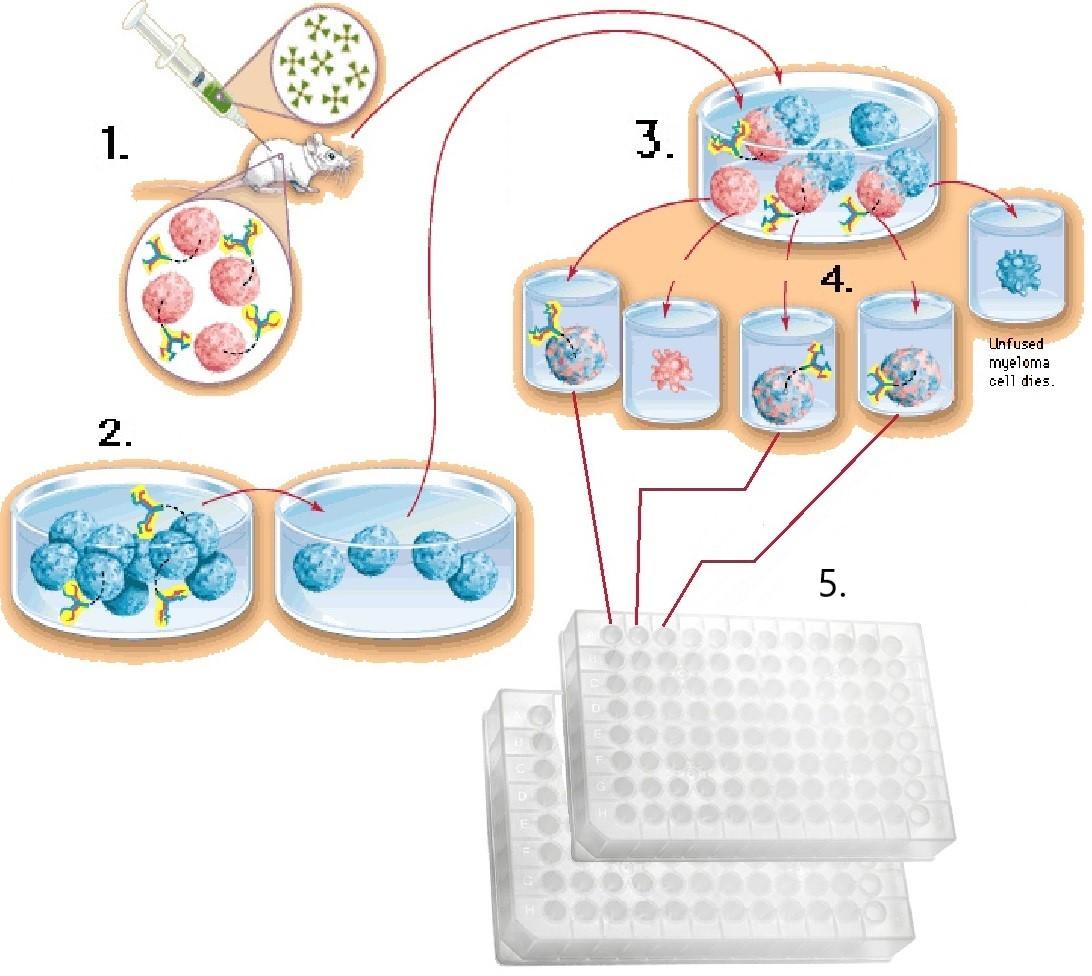


**Fig.7. Schematic diagram of experiment**

1. Making *antibody* for Ang-1
2. Test to the interactions between Tie2 – Ang-1 and αVß3 - Ang-1 with antibodies:
   1. Confirming Antibody – Ang1 Binding
   2. Association between Tie2 – Ang-1 and αVß3 - Ang-1 with presence of antibodies.
3. Select a specific antibody that blocks ONLY the association between αVß3 and Ang-1,
4. 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 48-49.**

1.Mouse is injected with Angiopoietin-1, 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 [[See more](https://cdn.stemcell.com/media/files/manual/MA28411-ClonaCell_HY_Hybridoma_Cloning_Kit.pdf), 4.1 Preparation of Myeloma Cells]

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.

5. Hybridomas that survived are then transferred to 96-well plates with HAT medium one cell in each well. Each plate acts as a *monoclonal* culture, each hybridoma in a well (inherit 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.

***[If the cells are stuck to the wells, then you should be able to use copies of these plates for Steps II through IV, much simpler.]***

***I do not know if the cell will be stuck to the wells, to be safe I will follow the ELISA protocol to prepare the ELISA plates instead.***

## Step II: Confirming Antibody – Ang1 Binding

**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](https://www.abcam.com/protocols/indirect-elisa-protocol)]

* + - 1. **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](https://www.abcam.com/protocols/indirect-elisa-protocol)]

* + - 1. **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

* + - 1. **Wash**:

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

1. **Adding secondary antibody**:

Secondary antibody with fluorescent tag (store-bought with green-fluorescent Alexa Fluor® 488 dye molecule: [See more](https://www.thermofisher.com/antibody/secondary/query/alexa+488)) is added to all Step II microplates. The secondary antibody binds to *Fc region* of primary antibody.

[[see more](https://www.thermofisher.com/us/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/imaging-basics/labeling-your-samples/different-ways-to-add-fluorescent-labels.html)]

1. **Wash**:

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

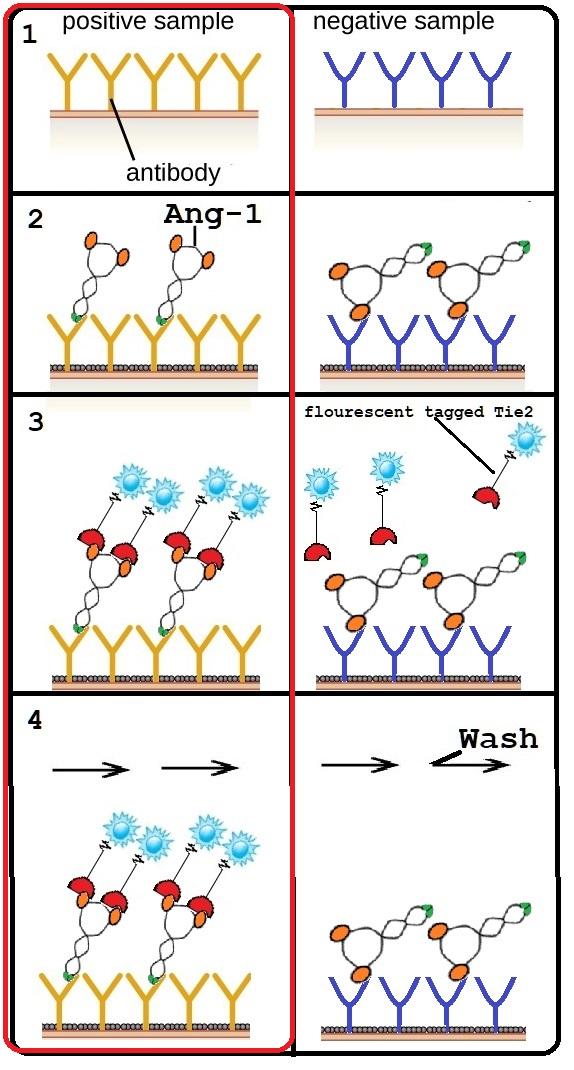
1. **Visualization:**

High energy light (UV) is used to read the ELISA plates. Plates emitting strong 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 “Step II: selected” accordingly to the selected plates:

For example: if ELISA well #1 emit a strong fluorescent, **Step I.6** well #1 is marked “Step II: selected”.

## In vitro: Association between Tie2 – Ang-1 and αVß3 - Ang-1 with presence of antibodies.

### Step III: Tie2 – Ang-1

**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.

Selected wells’ supernatants from **step II.7** is added to coat the fresh plates.

NOTE: Step III and IV started with the same set up. This step is repeated to prepare for Step IV.1

For example:

**Step I.6** marked “Step II: selected” well #1 supernatant is used to coat **Step III.1** fresh well#1

Well #2 and #3 are not marked therefore skipped

**Step I.6** marked “Step II: selected” well #4 supernatant is used to coat **Step III.1** fresh well#4… and so forth

[following general protocol: [Sandwich-ELISA](https://www.abcam.com/protocols/sandwich-elisa-protocol-1)]

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 [using [Protein Labeling Kits](https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-labeling-crosslinking/fluorescent-protein-labeling.html) 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**:

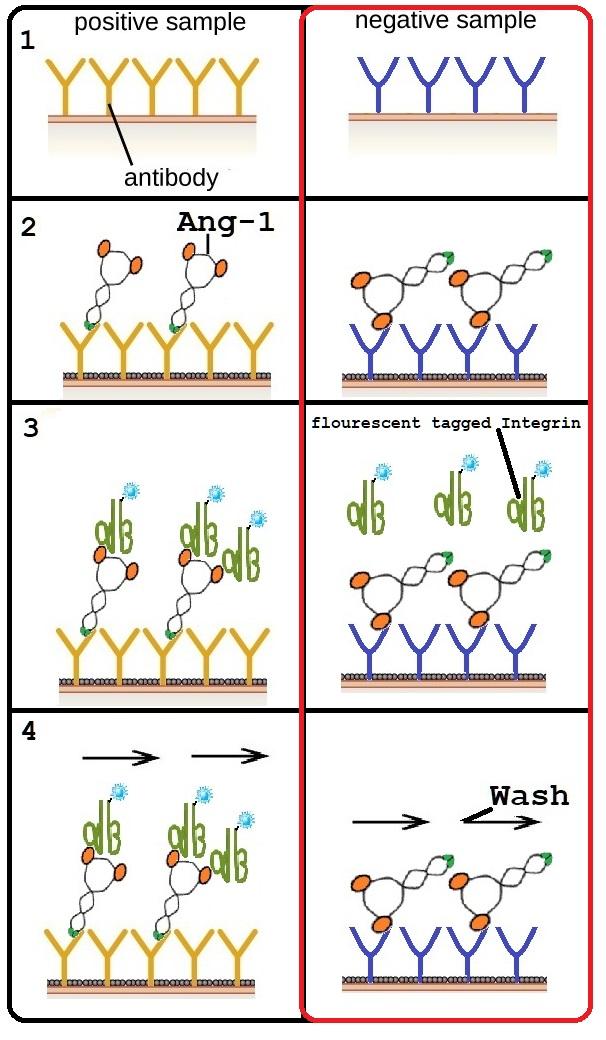
Using PBS to wash all the plates, unbound Tie2 is washed away.

5. **Visualization:**

High energy light (UV) is used to read the ELISA plates. Plates emitting strong 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 “Step III: selected” accordingly to the selected plates:

For example: if ELISA well #4 emits a strong fluorescence, **Step I.6** well #4 is marked “Step III: selected”.

### Step IV: αVß3 – Ang-1

**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 αVß3:**

Ectodomain Fluorescent-tagged Integrin αVß3 [using [Protein Labeling Kits](https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-labeling-crosslinking/fluorescent-protein-labeling.html) 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 αVß3. 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 αVß3

4. **Wash**:

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

5. **Visualization:**

High energy light (UV) is used to read the ELISA plates. Plates emitting strong 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 “Step IV: selected” accordingly to the selected plates:

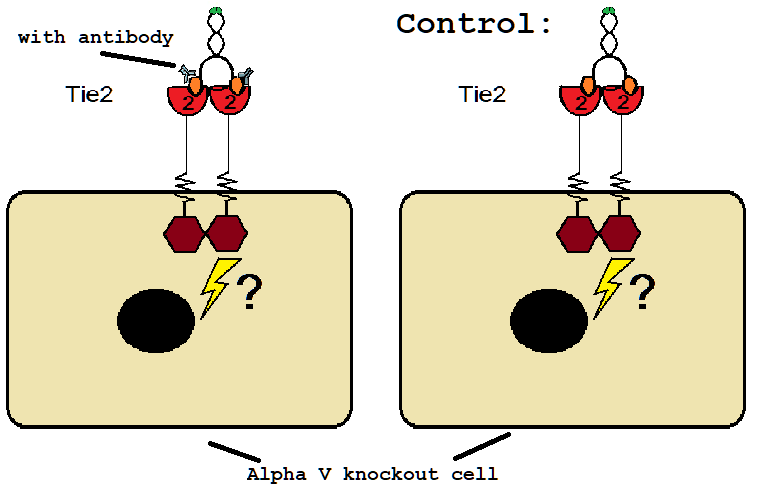
For example: if ELISA well #4 does not emit fluorescent, **Step I.6** well #4 is marked “Step IV: selected”.

## Step III-IV: results comparison

Since the results of both **step III** and **step IV** are documented in the copy made in **step I.6**, this step is about selecting wells that are both selected by step III and step IV (i.e. samples that are marked “Step III: selected” and “Step IV: selected”). The wells that are qualified for both steps will be marked “III-IV: selected” and their monoclonal antibody will be used in **step V, VI** and **VII**.

## (Optional) In vivo: Association between Tie2 – Ang-1 and αVß3 - 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 αVß3 expression to test Tie2 activation without integrin’s interference. Only knockout αV is enough to bringdown αVß3 complex

Knockout αV can be ordered through a couple companies [similar products: [See more](https://www.accegen.com/immortalized/49562.html)]

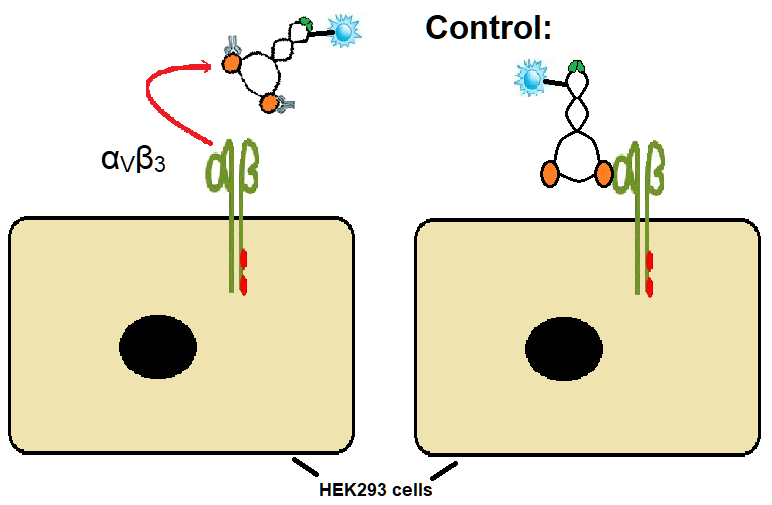
**Fig.13. In vivo: Confirming Antibody – Ang1 Binding with Tie2 in αV knock off cells**

In αV 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: αVß3 – 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](https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-labeling-crosslinking/fluorescent-protein-labeling.html) 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).

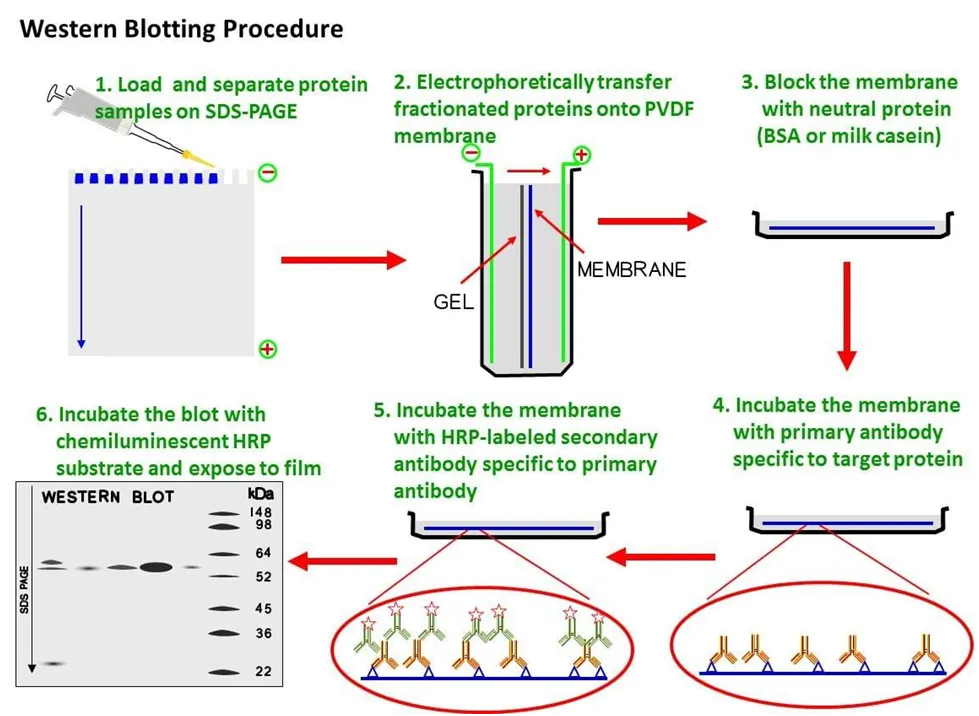
## Step V-VI: results comparison

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 αVß3

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

## Step VII: Measure Tie2 activity in presence of Ang-1 antibody and Integrin αVß3



**Fig.15. Western Blotting with Anti-phosphotyrosine immunoprecipitates as primary antibody51**

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

Each well column are loaded with the following compositions:

Reference columns:

Column 1: Tie2

Column 2: αVß3

Column 3: αVß3 +Tie2

Column 4: Ang-1

Column 5: Ang-1 + αVß3

Column 6: Ang-1 + Tie2

Column 7: αVß3 +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.

* + - 1. **Transfer to membrane:**

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

* + - 1. **Blocking:**

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

* + - 1. **Addition of primary antibody:**

00 (Ptyr) (store-bought: [See more](https://www.abcam.com/phosphotyrosine-antibody-py20-ab10321.html)) is added as the primary antibody. Anti-phosphotyrosine antibody recognizes tyrosine phosphorylated proteins (Tie2 autophosphorylation in this experiment 53. Then the membrane is washed off excess unbound primary antibodies by PBS buffer.

* + - 1. **Addition of secondary antibody:**

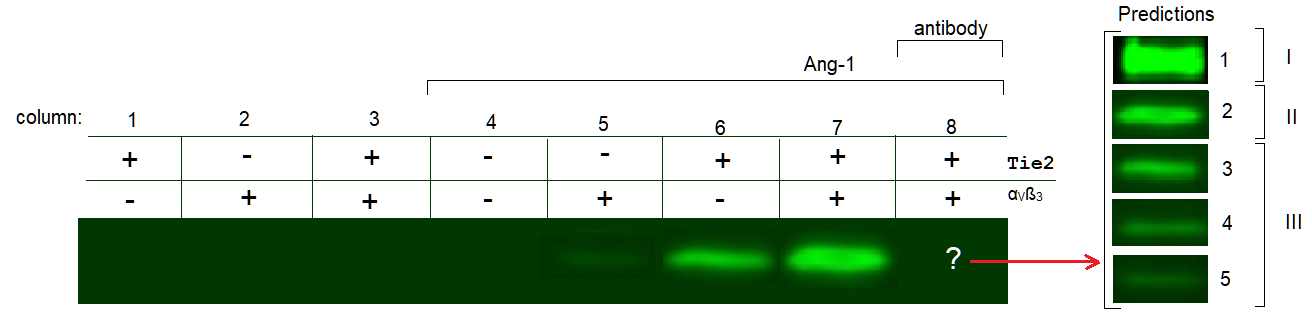
Secondary antibody tagged with fluorescent tag (store-bought with green-fluorescent Alexa Fluor® 488 dye molecule: [See more](https://www.thermofisher.com/antibody/secondary/query/alexa+488)) is added, then washed off any excess. This secondary antibody is specific to the primary antibody (like binding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

* + - 1. **Visualization:**

Expose the membrane to UV light to get the reading

# Discussion:

## 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/ αVß3 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/ αVß3 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/ αVß3 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 αVß3 by its ligand Ang-1 (in which αVß3 does not bind to Ang-1). The effects of Ang-1 on αVß3 and how that affects Tie-2 activation is not in the scope of this experiment.

If by blocking Ang-1 binding to αVß3 lowers Tie2 activation, it could become a new drug target. Thus, it can be coupled with other Tie2 inhibitors to create compounded medication.

# Glossary:

**αxßy**: 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

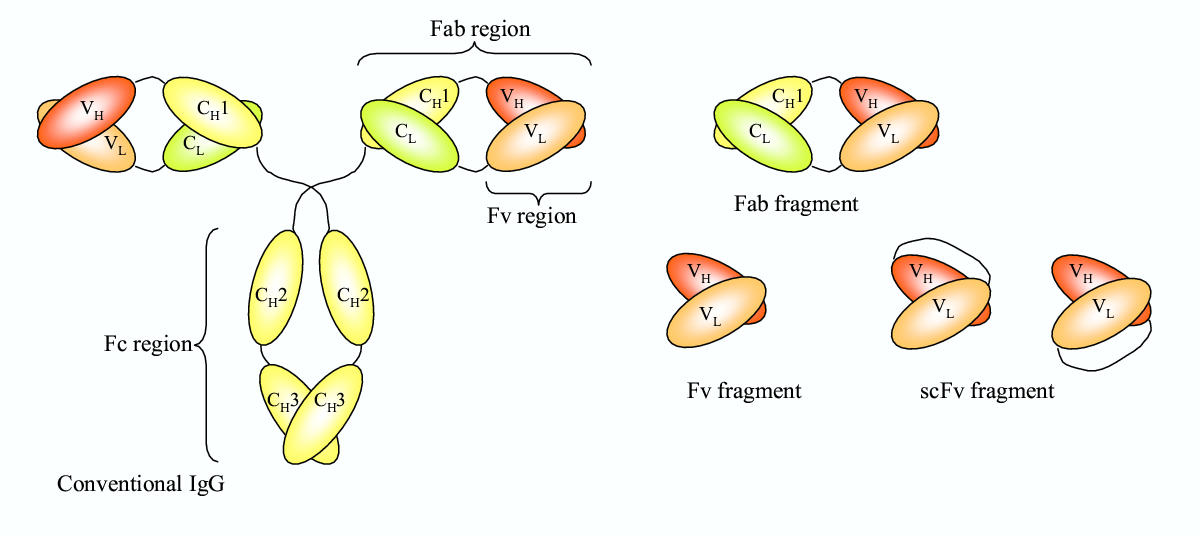
**Cardiomyocyte:** cells responsible for generating contractile force in the intact heart

**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](https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html)

**Endogenous:** within an organism

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

**Fc region of antibody:**

[See more](https://www.labome.com/method/Antibody-Structure-and-Antibody-Fragments.html).

**HAT medium:** is a selection medium for mammalian cell culture. [See more](https://www.thermofisher.com/us/en/home/life-science/cell-culture/mammalian-cell-culture/media-supplements/hat-supplement.html)

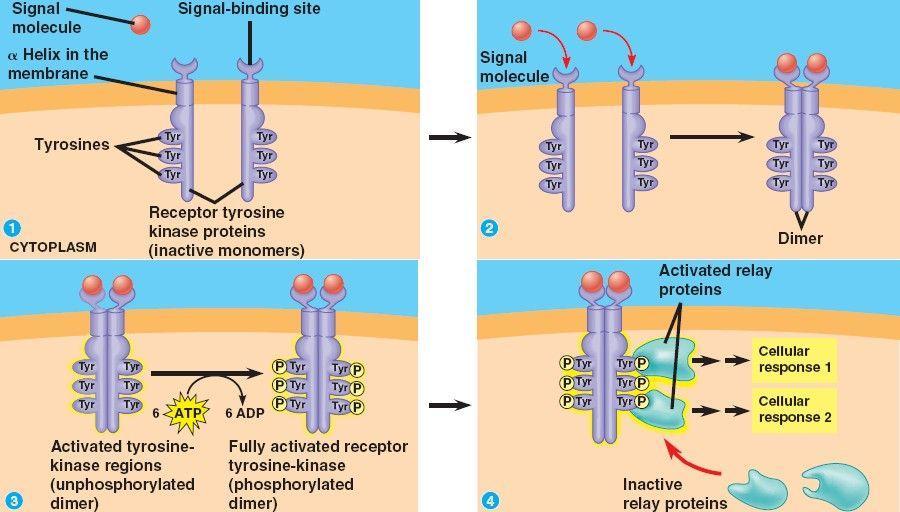
**Heterodimer dissociation:** in this context, it means the dissociation of Tie1-Tie2 complex

**Hybridoma**:  [B cell](https://en.wikipedia.org/wiki/B_cell), produces antibodies fused with immortal B cell cancer cells, a myeloma to produce a hybrid [cell line](https://en.wikipedia.org/wiki/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](https://www.thermofisher.com/order/catalog/product/AM9625#/AM9625)

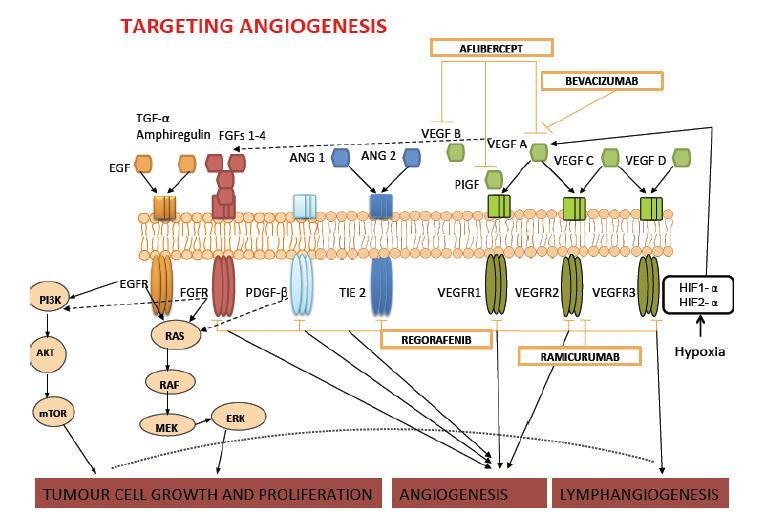
**Pulmonary edema:** excess fluid swelling in the lungs.

**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.”38

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

**Utero:** in the womb

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