

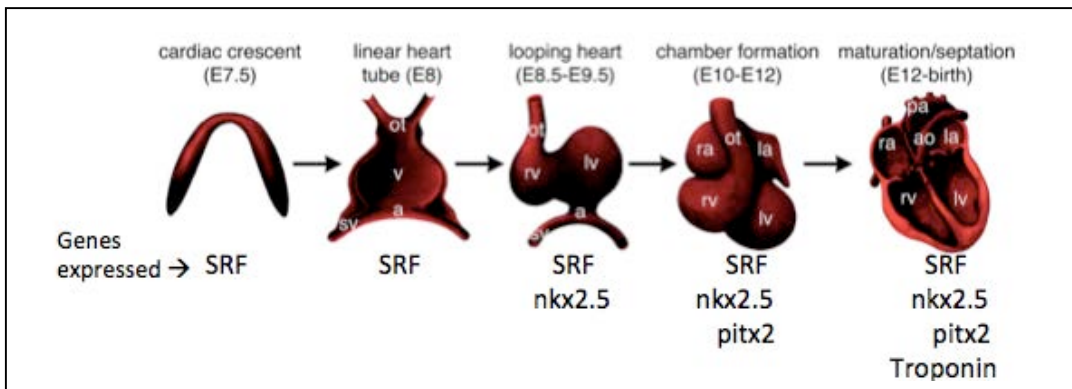
Solution key- 7.013 Problem Set 6- 2018

Introduction

The heart pumps oxygenated blood through the body and sends deoxygenated blood to the lungs for re-oxygenation. Heart disease is a major killer in the US. Malformations of the heart are a common birth anomaly. This makes understanding development and regeneration of the heart of great interest. This problem set focuses on mechanisms underlying cardiac development and strategies for repair.

The mouse heart develops over time as diagrammed in Figure 1 (*from Bruneau, 2002*) and explained below. (© Wolters Kluwer Health, Inc. All rights reserved. This content is excluded from our Creative Commons license. For more information, see <https://ocw.mit.edu/help/faq-fair-use/>)

- A group of cells form a crescent shaped region (on embryonic day 7.5 (E7.5))
- Future heart cells form a tube (E8)
- The tube loops (E9.5)
- Heart chambers form (atria (a) and ventricles (v)) (E10)
- Heart walls form their final fate: 'cardiac muscle', which beats (from E12 onward)
- Gene expression changes over time. Genes expressed at each stage are shown below.



Question 1 (5pts)

In order to understand when the cardiac muscle 'knows' what it is going to form, scientists performed an isolation experiment, where future ventricle cells (v) were removed at E7.5 and E8.5. These isolated cells (called 'explants') were allowed to grow and develop in the lab. The explants were assessed 6 and 5 days later respectively, when embryos from which they were removed would have reached 13.5 days old.

Days at which explants were isolated	Days for which explants were grown in the lab (until the embryos would be 13.5 days old)	Beating cardiac muscle formed?
E7.5	6 Days	No
E8.5	5 Days	Yes

- a) From this experiment, when do cells commit to form cardiac muscle? *E8.5 (0.25pts)*
- b) From this experiment, at what stage do heart cells differentiate? *From E12 → E13.5 (0.25pts)*
- c) What is the combinatorial gene expression code for heart development?
SRF+nkx2.5+pitx2+troponin genes expression makes the combinatorial code (0.5pts)

Question 1 continued

d) SRF, nkx2.5 and pitx2 are all transcription factors. Classify... *(0.25pts each or 0.5 total)*

- i.** SRF as ubiquitous/ regulatory (restricted)/ effector (cell-type specific) protein. **Circle one.**
- ii.** nkx2.5 as a regulatory (restricted) OR effector (cell-type specific) protein. **Circle one.**

e) You make mouse mutants that have homozygous loss-of-function mutations in nkx2.5, pitx2 and troponin genes. You observe the following changes in RNA of the developing heart at E13.5. **Note:** *the row showing the wild type is shaded.*

Mutants	RNA profile in the future heart
Control (wild type)	SRF, nkx2.5, pitx2, troponin
SRF	None
nkx2.5	SRF
Pitx2	SRF and nkx2.5
Troponin	SRF, nkx2.5, pitx2

Based on the table, draw a flowchart to account for how SRF, nkx2.5, pitx2 and troponin genes regulate one another

SRF → nkx2.5 → pitx2 → troponin
(0.5pts)

f) Interestingly, you identify a mutant that has the RNA for nkx2.5 but there is no Nkx2.5 protein. You examine the nkx2.5 RNA and see it is larger (in terms of the numbers of bases) than that observed in the wild type. **Give one plausible explanation for a lack of Nkx2.5 protein production.** *(0.5pts)*
-The introns are not spliced out perhaps due to a mutation in the splice donor or splice acceptor sites. This results in a longer mRNA in mutant cells compared to that in the wild type.
-Could also change promoter start site or polyA addition site.

g) BMP signaling is required for heart formation.

- BMP ligand binds to a receptor on the target cell membrane.
- The receptor is activated via phosphorylation.
- Activated receptor phosphorylates the Smad1 transcription factor.
- Phospho-Smad1 changes transcription of target genes, including SRF.

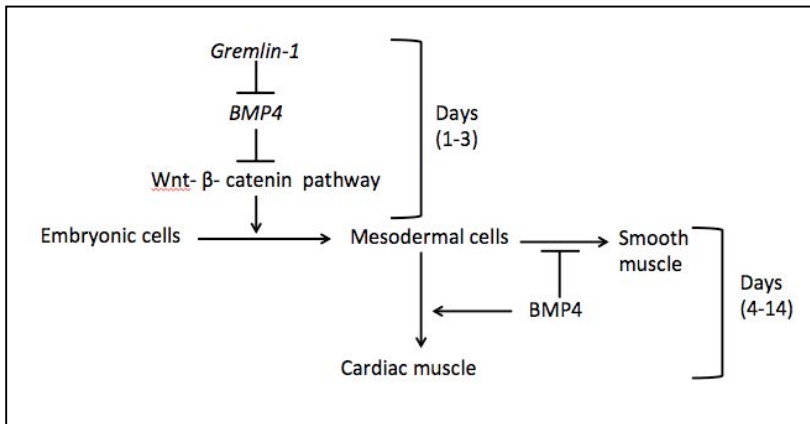
i. On what cells do you expect to find the BMP receptor? **Explain** your choice.
It should be on the surface of target cells that are determined to form the heart. (0.5pts)

ii. Where would phospho-Smad1 be localized: **Cytoplasm/ plasma membrane/ nucleus?** **Explain** your choice.
SMAD, being a transcription factor should be functioning in the nucleus of the target cell to regulate genes expression. (0.5pts)

iii. In a BMP receptor mutant lacking a signal sequence, briefly **explain** what would happen to heart formation?
It will not form since the target cells will not express SRF (and perhaps other proteins) and will therefore not have the right combinatorial code. (0.5pts)

Question 1 continued

Cardiac muscle is one type of contractile cell. Smooth muscle that lines blood vessels is another. The heart is largely cardiac muscle, but blood vessels entering and leaving the heart must make smooth muscle. There is therefore a choice of which muscle type is made, shown by the gene regulatory network below (*Reference: Umezawa et al PLoS 2008*).



- Gremlin-1 promotes Wnt- β catenin signaling by inhibiting BMP4.
- This promotes the formation of mesoderm cells from the embryonic cells
- Mesodermal cells either form cardiac muscle if BMP4 is active or skeletal muscles when BMP4 is inactive.

Predict whether **cardiac muscle/ smooth muscle/ neither/ both** will be formed if

- In a *Gremlin-1* homozygous null mutant? Neither (0.25pts)
- If the cells in the mesoderm have a heavily methylated *BMP4* gene promoter? Smooth muscle (0.5pts)
- In a constitutively active β -catenin? Cardiac muscle if you assume that BMP4 is present but smooth muscle if you assume that BMP4 is absent (0.25pts)

Question 2 (5pts)

There are five cell types in the heart:

- Cardiomyocytes (form cardiac muscle)
- Endocardial cells (line the heart chambers)
- Epicardial cells (form a surrounding sheath)
- Pacemaker cells (set heartbeat rhythm)
- Purkinje neurons (control contraction)

a) During heart development, cardiomyocytes and endocardial cells appear simultaneously. Is this consistent with **migration/ co-induction/ sequential induction**? Circle the correct option(s) and **explain** why you selected this option(s).

Co-induction: Since more than one cell type is formed at a place at the same time (0.25pts)

Migration: the cells may reach the correct location at the same time due to the directed movement (0.25pts)

b) Later, the other cell types appear. If you remove the cardiomyocytes, no pacemaker cells appear. Is this consistent with **migration/ co-induction/ sequential induction**? Circle the correct option(s) and **explain** why you selected this option(s).

Sequential induction since the appearance of pacemaker cells is dependent on the prior formation of cardiomyocytes (0.5pts)

Question 2 continued

c) Formation of the heart tube requires lengthening of the existing epithelium.

- i. What is an epithelium? *It is an outer sheet of cells. (0.25pts)*
- ii. Name three processes that can lengthen an epithelium, and briefly **explain** how each does so.
 - Epiboly: The cells of the tube lengthen or stretch out*
 - Convergent extension: Cells intercalate into the epithelial sheet forming the tube.*
 - Cell proliferation would increase number of cells and therefore length,**(0.75pts or 0.25 for each process)*

d) Predict the effect of each of the following events on the formation of the heart tube. **Offer a reasonable mechanism to account for your prediction.**

- i. Cells express a defective cadherin protein that lacks its extracellular domain.
Epithelial cells would not show the cadherin dependent homotypic cell – cell adhesion that is required to form the heart tube. The epithelium would be defective. The tube would be small, disorganized or non-existent. (0.5pts)
- ii. Cells are treated with nocodazole, a small molecule inhibitor of microtubule function.
This will influence the cytoskeleton and accordingly change cell shape. The tube may not form because the cells cannot elongate properly. Formation of the spindle in cell division will also be disrupted. So there would be fewer cells present and a smaller tube. (0.5pts)

e) The cardiac epicardium migrates into the heart region to set up a protective cell sheet around the developing heart.

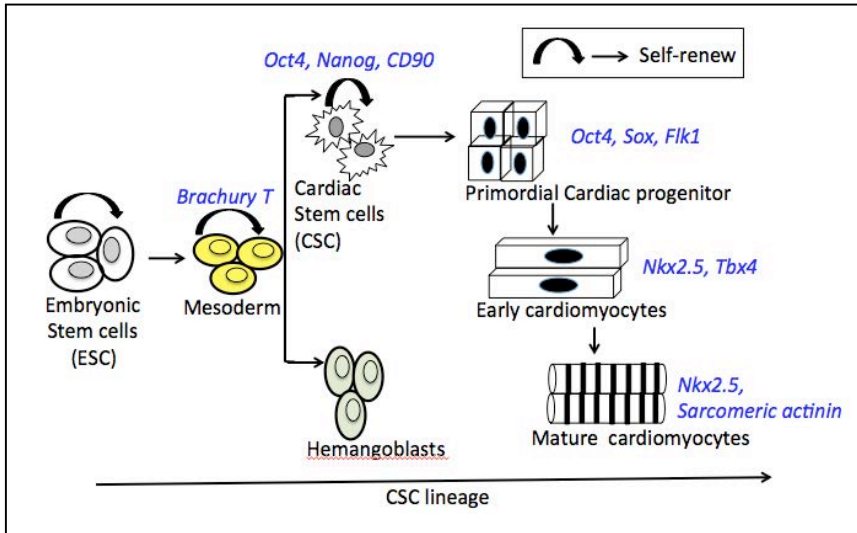
- i. What is the name for the cell state that can migrate? *Mesenchyme (0.5pts)*
- ii. What is the process called by which migratory cells convert to a cell sheet? Give two changes that occur during this process. *(0.5pts)*
Mesenchymal to epithelial transition MET. In this state, they acquire cell junctions and polarity.

f) Troponin protein is critical for heart muscle contraction and functions in cytoplasm. **Explain** what mutation of troponin gene that might give the results described below. Where multiple answers are possible, **list only one** for each of the following parts.

- i. Cells secrete Troponin protein.
Protein perhaps has the signal sequence (perhaps due to an insertion mutation in the gene) which causes its translation on the cytoplasm first and then on the ER membrane. (0.5pts)
- ii. Cell lacks the mRNA corresponding to the troponin protein.
Mutation in the promoter sequence, methylation of promoter. (0.5pts)

Question 3 (5pts)

Stem cells are being considered as a means to treat heart failure. Cardiac stem cells (CSC) can be prepared starting from embryonic stem cells (ESC) as shown in the following lineage. **Note:** Genes expressed in each cell type are italicized and shown in blue.



a) Are all the cells in this lineage likely to have **IDENTICAL** histone acetylation patterns? Why or why not? (1pt with 0.5 for explain)

Not identical. Histone acetylation is part of chromatin structure, and chromatin structure regulates gene expression. Since cell type corresponds to gene expression, this would change for each cell stage in the above lineage, we would expect regulation, including histone acetylation to change.

b) List **two** characteristics of the CSC that qualifies them as stem cells.

They can self renew by dividing symmetrically and/ or assymmetrically. Their assymetric cell division results in the formation of all cell types of the CSC lineage. (1pt)

c) CSC transcription factors include **Oct4 and Nanog** and the cell surface protein **CD90**. Which of these proteins would be most useful to purify living CSC from a mixed population that has multiple cell types by FACS (Florescence activated cell sorter)? **Explain** your choice.

CD90 since you can use an antibody (it is a multimeric , large protein that cannot get into the cells without disrupting their membrane) specific to this surface marker to purify the CSC population by FACS. (0.5pts)

d) **CSCs are also naturally found in the adult heart (at very low levels)**. You want to determine the half-life of CSC in mice by FACS analysis. What technique would allow you to do so? Briefly describe an experiment using this technique that will let you make the determination.

Pulse chase assay: You would label the NSC with bromodeoxyuridine (BrdU), which will incorporate in to the genome of the dividing CSCs. Then you remove BrdU label and add thymidine instead. You follow the BrdU labeled cells over time to trace ½ life of the cells, the entire CSC lineage and also the migration of the cells. (1pt)

e) Both ESCs and induced pluripotent stem cells (iPSC) can produce 'mesoderm' cells that are a precursor of CSCs. In terms of their origin, how does the ESC differ from iPS cells?

ESCs are derived from the cells of the inner cell mass (ICM) of a developing, early embryo. In comparison the iPSCs are formed by reprogramming the adult differentiate cells such as fibroblasts by adding a specific combination of transcription factors. (0.5pts)

Question 3 continued

f) In adults, CSCs reside in a defined region of the heart called a "niche". CSCs require growth factor ligands such as EGF, from the niche for cell division and differentiation. You analyze two mouse mutants: **Note:** Mutant Mouse strain 1 expresses EGF whereas Mutant mouse strain 2 does not.

Experiment A: You isolate CSCs from GFP-labeled mutant mouse strain 1. You transplant them into the cardiac region of mutant mouse strain 2 that is not GFP-labeled. After waiting for four days, you **do not observe** any GFP labeled differentiated cardiomyocytes in mutant mouse strain 2.

Experiment B: You isolate CSCs from GFP-labeled mutant mouse strain 2. You transplant them into the cardiac region of mutant mouse strain 1 that is not GFP-labeled. After waiting for four days, you **observe** GFP labeled differentiated cardiomyocytes in mutant mouse strain 1.

- i. Which of these is likely to have mutations in the genes that encode the EGF receptor?
Explain your choice.

Per Exp A: You take the Mouse 1 CSC and grow them in Mouse 2 . You see no green cells. This is likely because Mouse 2 does not have the EGF to cause the proliferation and differentiation of Mouse 1 CSC. So the niche of Mouse 2 is not normal. Per Exp B: You take the Mouse 2 CSC and grow them in Mouse 1. You see green cells. This is likely because Mouse 2 CSCs have the EGFR (although their Niche does not secrete EGF) whereas mouse 1 CSC lack the EGFR but their niche has the EGF. So Mouse 2 CSC (which have the EGFR) can grow in mouse 1 niche (that makes the EGF) but the reverse cannot happen. (No points)

- ii. Why did you use GFP-labeled cells?

To trace the cells derived from the transplanted cells (0.5pts)

Question 4 (4pts)

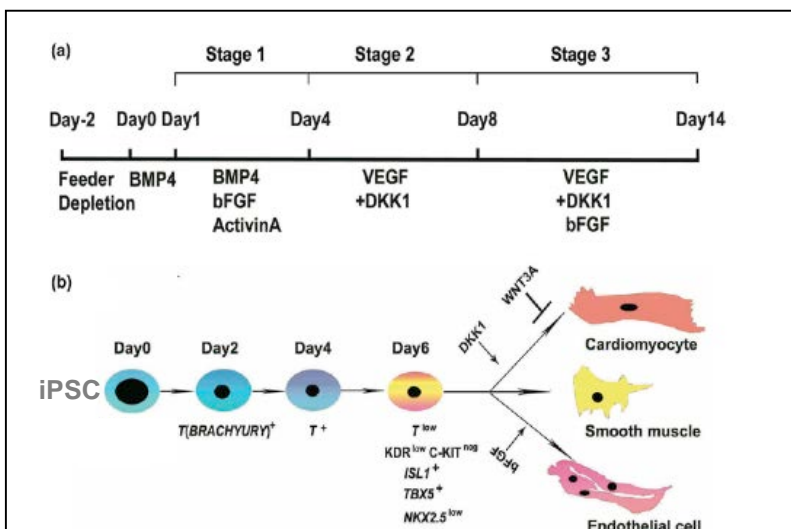
Cardiomyopathy refers to disease of cardiac muscle. The goal of regenerative medicine is to provide cells that can repair diseased cardiac muscle. You begin by preparing human cardiomyocytes from iPSCs cells.

- a) Starting with human skin cells, briefly **explain** how you would generate iPSCs.

You would insert a set of 3-4 Transcription factors into the fibroblasts to see if they revert back and acquire the pluripotent state. (0.25pts)

- b) Would you class iPSC as totipotent, pluripotent, bipotent? **Explain** your answer.

They are pluripotent since they can produce all cell types except placenta. (0.25pts)

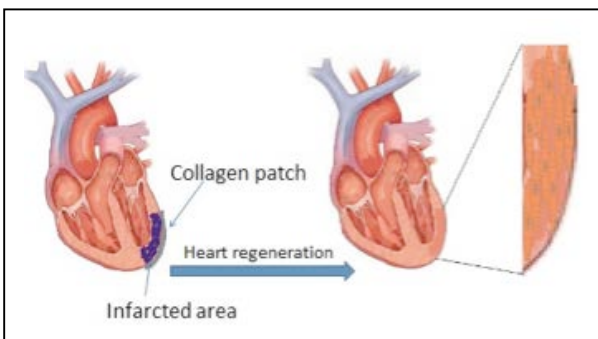


The lineage diagram below shows formation of cardiomyocytes from iPSC (**Reference:** Lu and Yang Stem Cell Research & Therapy 2011) **Note:** BMP4, bFGF, Activin A, VEGF, DKK and WNT3A are signaling factors. T(Brachyury), KDR, C-KIT, ISL1 and TBX5 are transcription factors. Based on the schematic:

- i. Which transcription factor(s) would you hypothesize is essential for initiating the progression towards cardiomyocytes? **T (BRACHYURY) (0.25pts)**

Question 4 continued

- ii. Give an experiment that would test your hypothesis. What would you expect if your hypothesis is supported? (1-2 sentences) *(0.5pts)*
You can knockout the gene encoding the transcription factor T (BRACHYURY). If you are successful, you would see no expected differentiated cells as a result.
- iii. Which transcription factor(s) are likely essential for promoting differentiation of cardiomyocytes, smooth muscle and endothelial cells?
The transcription factor DKK1 (for differentiation of cardiomyocytes). But T, KDR, C-KIT, ISL1, TBX5 also okay since they initiate differentiation of ALL three cell types (0.25pts)
- iv. Why are the signaling (growth) factors sequentially applied during the culture period?
Takes multiple steps and cell states between the iPSC and final fate. Sort of recapitulate embryogenesis. (0.25pts)
- v. If you wanted to maximize production of cardiomyocytes...
- What growth factor(s) would you apply at day 8? Justify each of your answers.
Dkk, promotes cardiomyocytes (0.25pts)
 - What would you NOT apply? Justify each of your answers.
Wnt3A, inhibits cardiomyocytes, bFGF, promotes endothelial cell formation. (0.25pts)
- vi. At the start of the scheme, what does 'feeder' refer to? Why would you want to deplete this before the differentiation scheme begins? *(0.5pts)*
Feeder is a layer of cells that promote iPSC growth. They produce LOTS of growth factors and you want to get rid of these so the specific scheme you have in mind will work effectively.
- c) How would you determine whether you had produced functional cardiomyocytes after the culture period? *You can do so by looking at the expression of cardiomyocytes cells specific genes. Other answers also acceptable such as checking morphology, proteins level, immunohistochemistry etc.*
- d) One approach to cure cardiomyopathy is to use tissue engineering. In the diagram below, a collagen patch is placed into the damaged area of a heart.



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- i. What is the function of the collagen patch?
Solid support for cells, factors that can be targeted to the correct place. (0.25pts)
- ii. Why is collagen a good choice of material?
Biocompatible, part of normal basement membrane, can be broken down by surrounding cells and removed when repair is complete. (0.25pts)

Question 4 continued

e) As a model for cardiomyopathy, you define a mouse mutant with some characteristics of cardiomyopathy, however, the phenotype is variable between animals. You decide to perform somatic cell nuclear transfer (SCNT) to clone animals with more uniform characteristics. For this use of SCNT you have the option of using the nucleus from **adult CSCs, embryonic CSCs or adult cardiomyocytes**.

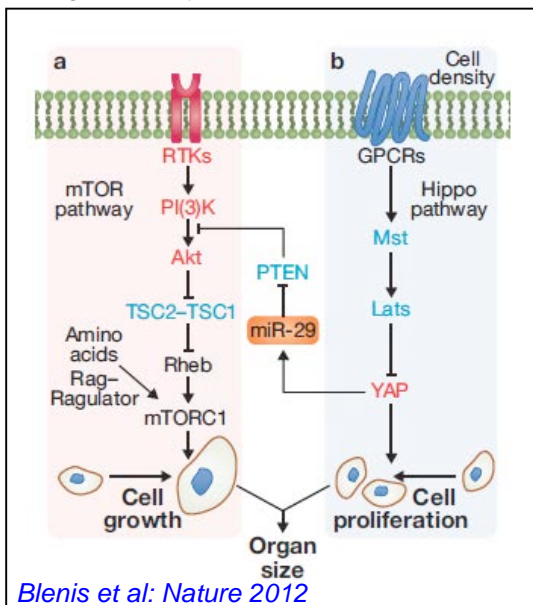
- i. Categorize the options as 1-3 with 1 being the best and 3 being the worst and provide an explanation **for your** classification. (0.25pts)

Embryonic CSCs will be the best choice since the methylation pattern are important for proper gene regulation and should be as close to the early embryonic state as possible for successful regeneration of animals by SCNT. This is followed by adult CSC and then adult cardiomyocytes.

- ii. 5-aza-cytosine (5-azaC) is a nucleotide that prevents DNA methylation. Recent results show that CSCs grown in 5-azaC are more efficient in animal cloning than untreated adult CSCs. How can you explain this? *The 5-azaC treatment demethylates and unwinds the DNA thus bringing the methylation pattern of the cell closer to the embryonic state.* (0.25pts)

Question 5 (1pt)

The following is a schematic of signaling pathway crosstalk that regulates the size of organs and may be altered in tumor growth and tumor size of many cancers. The major steps of the cross-talking pathways are outlined below. (Courtesy of Springer Nature Publishing AG. Used with permission.)



mTOR pathway:

1. Growth factors bind and stimulate receptor tyrosine kinases (RTKs).
2. RTKs can activate the PI(3)K–Akt signaling pathway.
3. The activated Akt, a serine threonine kinase, inhibits the TSC1–TSC2 complex, allowing Rheb to activate mTORC1.
4. In parallel, amino acids activate the mTORC1 pathway through a mechanism requiring the Rag–Ragulator complex.

Hippo pathway:

- 1 The binding of the ligand activates G-protein-coupled receptors (GPCRs), which activate Mst and Lats.

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2. YAP activity is modulated by phosphorylation of Mst and Lats. YAP upregulates miR-29, which in turn downregulates PTEN, an inhibitor of PI(3)K and Akt. So, the two pathways crosstalk and coordinate cell number and growth.

Consider the following **mutations** in different components of the signaling pathway.

- #1: A heterozygous gain- of function mutation that results in a constitutively active YAP.
- #2: A homozygous loss-of-function mutation in PTEN.
- #3: A constitutively active mTORC1

Question 5 continued

Complete the table for each of the above **mutations** relative to wild type reference cells that are treated with ligands that bind to RTK and GPCR. **Note:** Consider each mutation **independently**.

(Only Row 1 is graded: 1pt all or none)

Mutations	Oncogene/ tumor suppressor gene	mTORC1 active (Yes/No?)	YAP active (Yes/No?)	Cell growth (More/ less)?	Organ size (bigger or smaller)?
#1	<i>Oncogene</i>	<i>Yes</i>	<i>Yes</i>	<i>More</i>	<i>Bigger</i>
#2	<i>Tumor suppressor gene</i>	<i>Yes</i>	<i>No</i>	<i>More</i>	<i>Bigger</i>
#3	<i>Oncogene</i>	<i>Yes</i>	<i>No</i>	<i>More</i>	<i>Bigger</i>

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