Shh: Silencing the Hedgehog Pathway

"I'm so relieved," Ann said as she plopped down in the coffee shop booth where her friend Delores was reading e-mail on her laptop.

"Oh, Ann!? What did the doctor say?" Delores asked.

"Well, I do have skin cancer, but it's not melanoma. It's basal cell something. Anyway, it's very common and easy to treat," Ann reassured her.

"Is it genetic?" Delores asked, "or does it have something to do with that nice tan you showed off during your teens and twenties?"

"Well no one else in the family has had skin cancer." Grimacing, Ann added, "It's more likely I'm paying for my tan."

After Ann left, Delores searched for "basal cell cancer" on the Web. She wondered how her friend ended up with skin cancer. She found a 2004 paper by Athar and colleagues that explored BCC (basal cell carcinoma) and the effect of UV radiation. BCC, the most common kind of cancer, was linked to problems with the hedgehog signaling pathway. Exposure to UV radiation was one way to impact the pathway.

"More questions than answers," Delores sighed. She looked up "hedgehog signaling pathway" in Wikipedia. She found that this pathway controls cell division and is important in early development. The pathway was first discovered in fruit flies with a mutation that made them shorter and especially bristly. The researcher thought the fly larvae looked like hedgehogs.

Delores returned to the Athar article. The researchers divided mice into two groups and then exposed them to UV radiation. One group was given a drug called cyclopamine, a known antagonist to the hedgehog pathway, in their drinking water, and the other group got plain water. The mice that got the cyclopamine had many fewer BCCs at the end of the experiment.

"I wonder if they will give Ann cyclopamine for her BCC?" Delores thought as she closed her laptop.

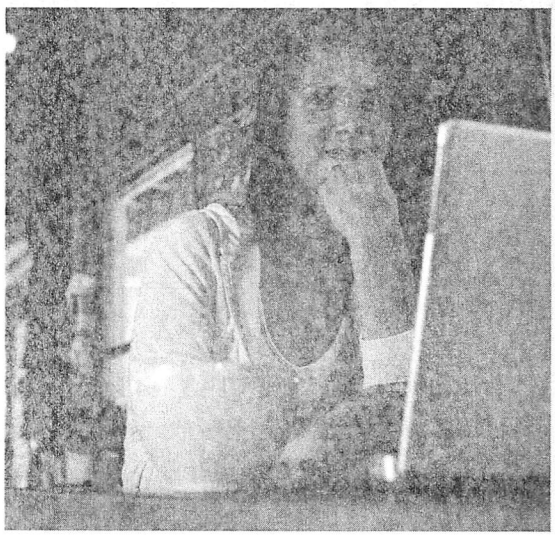

Figure 10.1 Using a laptop at a coffee shop.

CASE ANALYSIS

1. **Recognize potential issues and major topics in the case.** What is this case about? Underline terms or phrases that seem to be important to understanding this case. Then list 3–4 biology-related topics or issues in the case.

2. **What specific questions do you have about these topics?** By yourself, or better yet, in a group, list what you already know that is related to the case in the "What Do I Know?" column. List questions you would like to learn more about in the "What Do I Need to Know?" column.

What Do I Know?	What Do I Need to Know?

3. Put a check mark by **1–3** questions or issues in the "What Do I Need to Know?" list that you think are most important to explore.

4. **What kinds of references or resources would help you answer or explore these questions?** Identify two different resources and explain what information each resource is likely to give that will help you answer the question(s). Choose specific resources.

Core Investigations

I. Critical Reading: Cell Signaling Pathways

You should be familiar with the structure and function of proteins that have active sites, such as enzymes or antibodies. To complete this investigation, you should read Chapter 11: Cell Communication (specifically, Concepts 11.1 and 11.4) and Chapter 18: Regulation of Gene Expression (specifically, Concepts 18.4 and 18.5).

1. What is cancer? (Hint: Use of multiple sources for this definition, such as Cancerquest [http://www.cancerquest.org] in addition to the text, is recommended.)

2. What are some of the causes of cancer?

3. Interpret the graph in Figure 10.2 by answering the following questions.

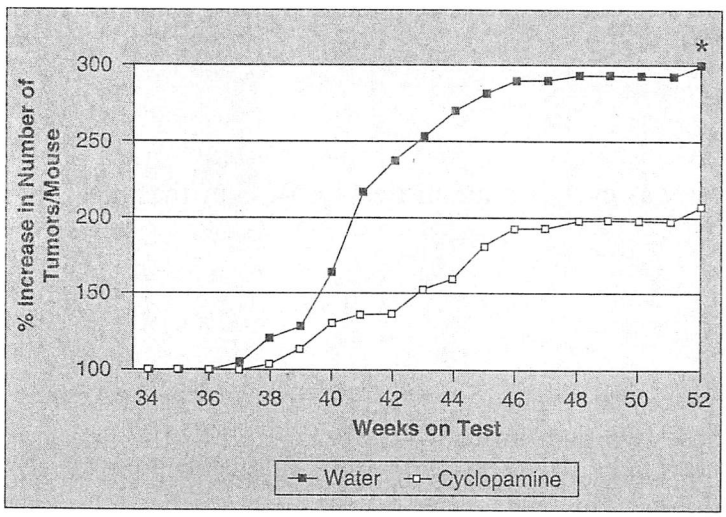

Figure 10.2 Effect of cyclopamine on BCC tumor formation in UVB-irradiated mice. (After Athar et al., 2004) (*Note:* The asterisk means the differences between the two treatments are statistically significant.)

a. On the basis of the shape of the curves, explain the patterns of tumor production in control and experimental mice in weeks 34–52.

b. What is the overall percentage increase in tumors for control versus experimental mice?

4. Use Figure 10.3 to answer the next two questions.

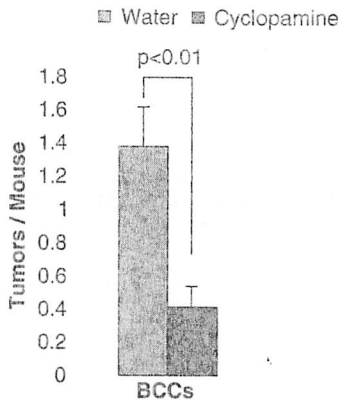

Figure 10.3 Average number of tumors per irradiated mouse with and without cyclopamine. (After Athar et al., 2004)

a. How effective was cyclopamine in treating BCC in the mice?

b. Which of these two data formats—the bar graph or the line graph—would be more effective in presenting the results of the experiment to the public? Which would be more effective for other scientists?

The hedgehog signaling pathway plays a crucial role in the development of many animal embryos. In addition, the hedgehog pathway controls regeneration of short-lived adult tissues, such as those in skin and blood. When the hedgehog pathway is active, transcription of proteins occurs in target cells followed by rapid division of those cells. The hedgehog pathway is also active in BCC and several other cancers.

The *hedgehog* gene family codes for signaling proteins that serve as ligands binding to receptors in nearby target cells. These proteins activate the hedgehog pathway in the target cells. The hedgehog pathway in the target cells has two membrane proteins named Patched (Ptch) and Smoothened (Smo), as well as several intracellular proteins.

When Shh (Sonic hedgehog) ligand binds to Ptch, then Smo is activated, the signal is transduced, and transcription and cell division result. In the absence of the hedgehog signaling protein, Ptch inhibits Smo, no signal is sent to the intracellular components of the hedgehog pathway, and thus transcription and cell division do not occur. Smo and the subsequent intracellular pathway may also be turned on by mutations that inactivate Ptch.

5. Is the hedgehog signaling pathway a local or long-distance type of signaling? Explain.

6. Examine Figure 10.4 and identify which molecules are involved in reception, transduction, and response in the hedgehog pathway.

7. The mechanism of the activation of Smo by the hedgehog ligand binding to Ptch is not completely understood. However, the model shown in Figure 11.11 in the text shows a pathway with two membrane proteins, similar to the arrangement of membrane proteins in the hedgehog pathway. In this model, cell signaling is involved when the ligand binds to the first receptor protein, activating the G protein. The G protein then activates the second membrane protein, which transduces the signal to the interior of the cell. Explain how this mechanism might be applied to the two membrane proteins in the hedgehog signaling pathway.

8. As scientists evaluate new data, they frequently have to revise their models. Because we know that Ptch is an inhibitor of Smo and G protein is not involved, revise the model in Figure 11.11 to incorporate this new information.

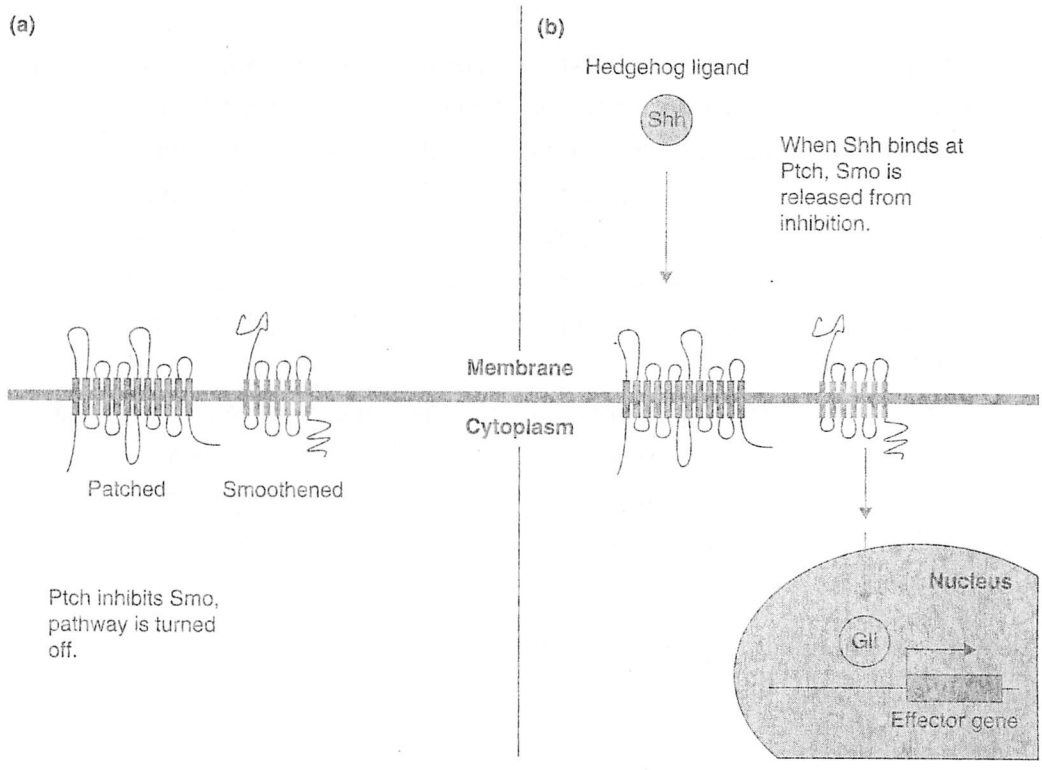

Figure 10.4 Schematic diagram of the hedgehog signaling pathway in vertebrates. (a) The target cell without the hedgehog ligand. Patched and Smoothened are transmembrane proteins embedded in the plasma membrane. Patched inhibits Smoothened and the pathway is turned off. (b) When the hedgehog ligand Shh joins with Patched, Smoothened is released from inhibition and the pathway is turned on. (Weitzman, 2002)

9. Cyclopamine is a known antagonist of Smo. Describe how cyclopamine reduces the number of BCCs in UVB-irradiated mice.

The hedgehog signaling pathway is active in the early embryo during development of the neural tube, motor neuron specification, left-right symmetry, body plan, limbs, and retinas (Matlack et al., 2006).

In the 1950s, sheep feeding on the corn lily (Veratrum spp.) in mountain pastures gave birth to a number of lambs with only one eye. The number of cyclopean lambs (named for the one-eyed Cyclops of Greek mythology) were explained when the compound later named cyclopamine was discovered in the corn lily. To see an image of cyclopia in sheep, go to http://teratology.org/jfs/NaturalTeratogens.html.

10. Explain how a failure to have cell division occur at a critical time during development could lead to lambs with one eye. See Concept 18.4 on critical events in the development of left-right symmetry and body plan.

II. Phylogenetics of the Hedgehog Gene Family

Nobel Prize researchers Christiane Nüsslein-Volhard and Eric Wieschaus investigated fruit fly mutations in order to make sense of the role of genes active in the development of fly embryos. They mutated one gene—later named *hedgehog*—that resulted in dense spines in shortened fly larvae.

Homologous *hedgehog* genes were later discovered in vertebrates. After these genes were sequenced in several different kinds of animals, they were compared and used to determine phylogenetic relatedness. If you have not yet studied phylogenetic classification, you may want to read Chapter 26 before completing this investigation. Consider the phylogram in Figure 26.12 in your text as you answer the following questions.

1. What species is used as the outgroup for the *hedgehog* gene in this phylogram? Provide a reason for using this species.

2. What does the phylogram tell us about the *hedgehog* gene in mammals and birds as compared to the *hedgehog* gene in mammals and amphibians?

Consider the ultrametric tree in Figure 26.13 in your text as you answer the following questions.

3. Does this ultrametric tree provide information about the rate of change in the *hedgehog* gene for these animal groups? If not, what information can be inferred from this tree?

4. List all the pairs of animal groups that share a more recent common ancestor than humans and birds.

The field of developmental biology is changing as scientists use molecular and biological approaches to investigate evolution questions. Since the discovery of classes of conserved regulatory genes or *Hox* genes, the new phrase "evo devo" has been used to refer to the science of evolutionary developmental biology. (See Concept 21.6 in your text.)

5. How could phylogenetic and ultrametric trees help inform researchers interested in designing experiments to study the hedgehog pathway?

After viewing the *hedgehog* phylogram in Figure 26.12, two researchers decide to look more closely at the relationship between hedgehog proteins found in animals. They obtain sequence information from two invertebrates and two vertebrates and choose to limit their study to a portion of the hedgehog protein produced by a highly conserved region of the *hedgehog* gene. The hedgehog proteins were produced by the gene Hh (hedgehog) in invertebrates and Shh (sonic hedgehog) in vertebrates.

The following amino acid sequences were produced from a conserved region of a gene in the *hedgehog* family of genes.

Scorpion Hh	DGPHAINSLH	YEGRAVDITT	SDRDRSKYGM	LARLAVDAGF	DWVYYESRAH	IHCSVKSESA
Human Shh	DGHHSEESLH	YEGRAVDITT	SDRDRSKYGM	LARLAVEAGF	DWVYYESKAH	IHCSVKAENS
Octopus Hh	QGHHAPTSLH	YEGRAVDITT	SDRVRSRYGM	LARLAVEAGF	DWVYYESRSH	IHCSVRSDSL
Chicken Shh	DGHHSEESLH	YEGRAVDITT	SDRDRSKYGM	LARLAVEAGF	DWVYYESKAH	IHCSVKAENS

6. Which of these organisms are invertebrates and to what phylum does each belong?

7. Which are vertebrates and to what class does each belong?

8. Based on the preceding limited amino acid sequences, which animal has the hedgehog protein most like the one in the human?

9. Which organism has the most differences in the amino acid sequence compared to the human hedgehog protein sequence?

10. Would you have predicted the answers to questions 8 and 9? Why or why not?

III. Critical Reading: Stem Cells and Gene Expression

Chapters 18 and 20 explore the basic question of how cells with the same DNA can become different cell types. Cell biologists cite differential gene expression in cells as the explanation. They are working with stem cells to better understand the regulation of gene expression in both developing and adult organisms. Cell signaling pathways play a critical role in this gene regulation, but it is important to note that pathways such as the hedgehog pathway have different roles in embryonic and adult stem cells.

With the exception of gametes, a complete set of chromosomes is found in all cells in the human body. However, not all genes are expressed in each cell. The proteins necessary for cell function depend on the location and function of a particular cell in the body as well as the specific conditions the cell confronts during its survival in the body.

1. Specify the location of a cell in your body that:
   a. contains genetic information on eye color and the production of insulin.

   b. expresses eye color.

   c. produces insulin.

Over the last century, cell determination in the developing embryo has been closely observed. New methods and tools enable modern scientists to probe this process at the molecular level.

2. What is the molecular definition of *determination*?

3. What molecules provide the earliest evidence that a cell is committed to a particular cell fate?

Stem cells are distinct from most cells in animals because they retain the ability to divide and remain relatively undifferentiated. Under certain conditions, however, stem cells divide and a subset of the new cells differentiates into specific cell types.

4. What are the major differences between stem cells found in embryonic tissue and those found in adult tissues?

The current interest in stem cells for regulation of gene expression is tied to providing potential new therapies for treatment of diseases such as the use of hedgehog pathway antagonists.

Current research with adult stem cells has provided some unexpected results (Figure 10.5).

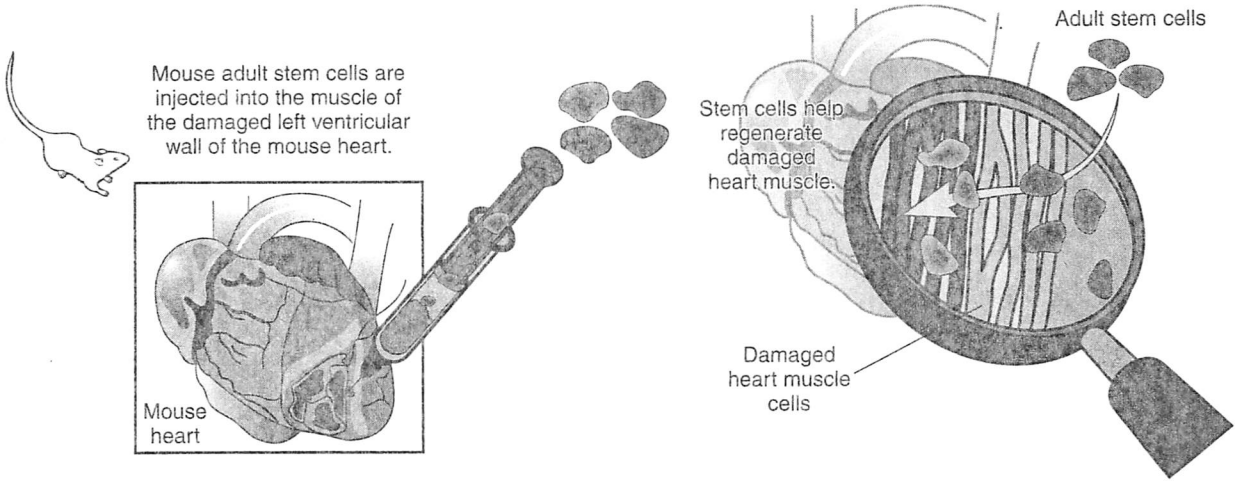

Figure 10.5 Mouse bone marrow stem cells injected into a damaged mouse heart resulted in new heart tissue. The bone marrow stem cells appear to secrete factors that promote regeneration.

Reference for image:
Stem Cell Basics: What are the potential uses of human stem cells and the obstacles that must be overcome before these potential uses will be realized? 2006. In *Stem Cell Information* (World Wide Web site). Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services (cited Tuesday, June 19, 2007). Available at http://stemcells.nih.gov/info/basics/basics6.

5. What kinds of cells can adult bone marrow cells give rise to?

6. Describe why human adult stem cells from bone marrow are used to treat patients who have undergone radiation treatments.

7. Provide an example of how treatment with cultured embryonic stem cells could be used to supply cells for the repair of damaged or diseased organs in human patients.

8. Use of adult stem cells is well accepted; however, these cells have limited use as donor cells. Human embryonic stem cells have greater potential uses in a wider variety of tissues; however, the use of embryonic stem cells raises ethical and political issues. Identify two concerns an individual might have with the use of embryonic stem cells.

IV. Investigating the Hedgehog Pathway: Antibodies as Research Tools

Scientists ingeniously design research tools based on *in vivo* processes of biological systems. For example, PCR is a technique utilizing the enzyme DNA polymerase to initiate the synthesis of a minuscule DNA sample. Likewise, Western blots and immunohistochemistry are techniques utilizing the highly specific binding of antibodies with target molecules to act as molecular probes in cells and tissues.

1. Explain how an antibody is able to recognize a specific antigen. (Include an explanation of an epitope in your answer.)

Antibody Techniques

Antibodies can be used to find, bind, and tag a specific molecule of interest. Antibodies are Y-shaped molecules, with the tips of the Y containing unique amino acid sequences that bind the antigen. These variable portions of antibody molecules convey the high specificity for a target molecule. The large tail or base of the Y is much less variable. In fact, all antibodies within a species have tail regions that are very similar in sequence and shape. By inserting compounds that fluoresce, produce radiation, or produce a color change in the tail region of these molecules, researchers can use antibodies as marker molecules.

Often, researchers use two different antibodies: a primary antibody for targeting the molecule of interest and a secondary antibody with active sites to bind the primary antibody's tail and act as a marker.

Western blots are used to detect the presence of a known protein in a given sample. The proteins are first separated by molecular weight using gel electrophoresis. Next, the proteins are transferred from the gel to a nitrocellose membrane in a process called blotting. The nitrocellulose membrane is then incubated with a primary antibody that combines with the protein of interest. Then an enzyme

coupled with a secondary antibody is used to produce a detectable color change when the protein of interest is present. The intensity of the color change indicates the quantity of the protein.

Immunohistochemistry uses antibodies to detect the presence of specific molecules, usually proteins, within tissues and cells. Thin sections of a biological sample are fixed to a glass slide, incubated with primary and secondary antibodies, and examined microscopically. Secondary antibodies used in immunohistochemistry are frequently fluorescent, in which case a fluorescent microscope is used to read the results.

2. Briefly describe what you can learn about a target protein by using each of these two techniques.

In 1996, it was discovered that a mutation in the *Patched* gene in the hedgehog pathway was involved in almost all cases of basal cell nevus syndrome, a rare hereditary syndrome of birth defects and multiple BCC starting early in life. This autosomal recessive mutation was identified in families affected by the syndrome. The *Patched* mutation resulted in a nonfunctional Patched protein.

3. Refer to the hedgehog pathway diagram (Figure 10.4) and explain what happens when the Patched protein is nonfunctional.

4. How could this lead to cancer?

As soon as the hedgehog pathway was implicated, researchers began looking at inhibitors that might serve as chemotherapy for this common cancer. Cyclopamine, the plant teratogen known to interfere with the hedgehog signaling pathway in early development, showed potential as a cancer treatment.

The exact mechanism by which cyclopamine inhibits hedgehog pathway signaling has been a topic of controversy. One hypothesis was that cyclopamine prevented the secretion of Shh from Shh-producing cells. The protein was expressed but without its normal cholesterol addition. As a result, Shh could not leave the cell to act as a signaling protein.

5. If you were asked to test this hypothesis, which technique do you think would be more useful—Western blots or immunohistochemistry? Explain your choice.

The Following Experiment was Designed to Test the Effect of Cyclopamine on Shh Secretion

Chick embryos in embryonic Day 3 (cells known to secrete Shh) are divided into cyclopamine-treated groups and control groups. After an established exposure time, embryos from both groups are sacrificed and thin sectioned for immunohistochemistry testing. Samples are incubated with primary, then secondary, antibodies per an established protocol. Under fluorescent microscopy, tissues are assessed for the presence and location of Shh.

Table 10.1 Antibodies for Hedgehog Proteins

Order Number	Protein	Tissue Specificity	Antibody Type and Source
1223	Hh	Drosophila	*Anti-Hh* goat, polyclonal
1224	Hh	Drosophila	*Anti-Hh* rabbit, polyclonal
2011	Ptc	Drosophila	*Anti-Ptc* goat, polyclonal
2624	Ptch	Mouse, rat, human, chicken	*Anti-Ptch* rabbit, polyclonal
2680	Ptch	Mouse, rat, human	*Anti-Ptch* goat, polyclonal
2681	Ptch	Mouse, rat, human	*Anti-Ptch* goat, polyclonal
2626	Ptch	Mouse, rat, human, chicken	*Anti-Ptch* rabbit, polyclonal
4235	Shh	Mouse, human	*Anti-Shh* mouse, monoclonal
4257	Shh	Human	*Anti-Shh* rabbit, polyclonal
4278	Shh	Mouse, human	*Anti-Shh* rat, monoclonal
4279	Shh	Mouse	*Anti-Shh* goat, polyclonal
4284	Shh	Mouse, rat, human, primate, chicken, cat	*Anti-Shh* goat, polyclonal
4286	Shh	Mouse, rat, human, zebrafish, Xenopus	*Anti-Shh* rabbit, polyclonal
3511	Smo	Drosophila	*Anti-Smo* mouse, monoclonal
6766	Smo	Mouse, rat, human	*Anti-Smo* rabbit, polyclonal
6788	Smo	Mouse, rat, human	*Anti-Smo* goat, polyclonal
6789	Smo	Drosophila	*Anti-Smo* goat, polyclonal

Based on a page from a research supply company catalog.

6. Consider the antibodies for hedgehog proteins listed in Table 10.1. Which one of these primary antibodies would you choose for the experiment described earlier? Why?

7. The secondary antibody includes a fluorescent marker in its tail region. Why?

The results of this experiment showed no difference between the test and the control specimens in the amount of fluorescence inside and outside the cells.

8. What can you conclude from these results?

New evidence suggests that cyclopamine's effect on the pathway may be caused by inhibiting Smoothened. Therefore, even in the presence of Shh, no message is transduced to the nucleus by Gli, and therefore there is no cellular response.

9. Consider the use of cyclopamine as a chemotherapeutic agent in cases of spontaneous BCC. Researchers discovered that this cancer results from a mutation of *Patched*. If cyclopamine was approved for human use, would you recommend it for these cases of BCC? Why or why not?

Additional Investigations

V. Open-Ended Investigations

To learn more about the hedgehog pathway, you could explore the Hedgehog Pathway Signaling Database (Ramirez-Weber, 2006), which contains relevant information, images, and references to research articles. You may wish to form a group to develop a proposal for a new investigation. For example:

- Explore a known hedgehog pathway antagonist other than cyclopamine.
  - How does this antagonist disrupt the pathway?
  - Does the antagonist have potential as a chemotherapeutic drug?
  - What organism produces this antagonist molecule and how is it useful in that organism?
- Choose one of the molecules in the pathway and compare the genes using Biology Workbench.

Note: Your instructor could set up a proposal peer review process in your class, simulating what is done by major funders such as the National Science Foundation and the National Institutes of Health.