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Augmenting Bone Regeneration: Structure, Function, and Dysfunction of the Skeletal System

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Introduction and Rationale

An insightful quote often credited to the Nobel Prize-winning physicist, Sir William Bragg, suggests that *"the important thing in science is not so much to obtain new facts as to discover new ways of thinking about them."* In my classroom, a version of this sentiment is imbued in all learning; I emphasize that we learn not simply to gather and consider information, but more so to apply new information to improve our lives. This is particularly evident in the structure of my Anatomy and Physiology course. Each unit of the course is organized around the same central idea: the human body is comprised of *structures* whose compositions dictate the *functions* they can perform and thus, *dysfunctions* result from changes in structure – structure, function, dysfunction. In this way, students can see that there are reasons for the data and information they gather about structure and function (beyond just having the information). This information is critical for providing insight about dysfunctions, and even more useful where it allows us to develop new or improved solutions for dealing with particular *dysfunctions*.

In essence, my Anatomy and Physiology course seeks to demystify (for students) disease and medical intervention by grounding knowledge in the firm foundation of structure-function-dysfunction. When students fully appreciate this foundation, they are able to use it as a platform from which to jump to new ideas. – again, from *new facts* to *new thinking*. Effective medical interventions – from common antibiotic treatments to groundbreaking gene-based therapies – stem from the idea that by better understanding bodily structures and processes, humans can take advantage and manipulate biological systems to enhance health outcomes.

As an example, consider the following example from the digestive system. Even before we begin the digestive system unit, many students are familiar with the term *heartburn*. However, if pressed about the condition and its possible causes, most students might at best manage a vague description of a painful dysfunction associated with heart muscle. A handful of students might be able to characterize heartburn as a problem associated with the digestive tract, but even they generally do not present a coherent and/or reasonable theory of the case. In terms of structure-function-dysfunction, heartburn, usually due to gastroesophageal reflux, is explained thusly; in the stomach, hydrochloric acid mainly functions to activate the enzyme pepsin (necessary for protein digestion) and to eliminate microbes. The relatively thick-walled structure of the stomach and the presence of bicarbonate usually prevent the stomach lining from

hydrochloric acid damage. The thin-walled, bicarbonate-free structure of the esophagus cannot adequately serve the same protective function. Consequently, a weakened gastroesophageal sphincter muscle for example, can result in backflow of acid into the esophagus causing pain and destroying the esophageal lining (Kumar, Abbas, & Aster, 2015). Fortified with an understanding of structural basis of the dysfunction, students are now able to understand, describe, and even *predict* the types of structural remedies that might restore normal function (e.g. ingesting compounds to neutralize the acid, surgically implanting foreign objects to reinforce the sphincter, surgically positioning wall of the stomach to support the sphincter, etc.)

The goal of the course is to help students see the body less so as a perplexing collection of opaque functions and confusing glitches, and more so as a concrete and knowable system. Deviations from optimum healthfulness – injury, disease, aging, etc. – can be better understood (and eventually remedied) as knowledge of the body's structure deepens. More straightforwardly, I push my students to embrace the idea that we explore the parts of the body, so we can know how those parts work; because it is by understanding how the parts work, that we might be able to address any problems that arise. To allude again to Bragg, we continually gather new facts to drive new thinking: new understanding and new solutions.

Throughout the Anatomy and Physiology course, the structure-function-dysfunction paradigm is applied to the various organ systems of the body. The curriculum unit presented here is to be a short segment taught within a broader unit of the course that covers the structure, function, and dysfunction of the skeletal system. Prior to engaging with specific content of this curriculum unit, students will have been introduced to and explored the idea that the structures of the skeletal system cooperate to serve several critical functions; interaction between the muscular system and the skeletal system allows the bones in the body to move; the axial skeleton, consisting of the skull, sternum, ribs and vertebral column, provides protection for the vital organs and soft tissues such as the brain, heart, and spinal cord; bones and cartilage, the only two rigid and dense parts of the body, provide shape and structure, maintain the body upright, and provide a framework to which all other soft tissues (muscle and organs) can attach; bones are the site of blood cell production; bones are an important site for mineral storage, especially calcium.

The curriculum unit presents a number of diseases and medical conditions that result in bone loss as representative disorders that students can explore in order to gain a better understanding of normal and abnormal functioning of the skeletal system. Osteoporosis, for example, is a skeletal disorder characterized by a loss of bone mass and decreased bone mineral density. The deterioration in microarchitecture of bone results in an increased risk of fracture. The curriculum unit establishes a foundation of normal bone development and function from which students will explore various skeleton-related dysfunctions alongside remedies and/or interventions; therefore, the curriculum unit reinforces students' understanding of the skeletal system by challenging them to thoroughly consider what happens when something goes wrong with it. With a more substantial understanding of the underlying science (molecules, mechanisms, pathways and the like), students will be able to rise to the curriculum unit's challenge of considering and grappling with risks, consequences, and ethical considerations associated with humans' abilities to manipulate human biology in these specific ways.

Demographics

My school is a high-poverty (100% qualify for free and reduced-price meals at school) neighborhood (non-application) high school in Washington, DC. The student body is predominantly black (62%) and Hispanic/Latino (36%). There is a significant international presence at the school. Several West and East African countries including Cameroon and Ethiopia are represented and many of the Hispanic/Latino students emigrated from Mexico and Central America. The school is in the midst of implementing an International Academy (starting with the lower grades), so the newcomer population is set to increase in the years to come. 30 percent of the student population are classified as English Language Learners (ELL). There are several of my students whose primary language is not English. In school, they generally communicate with peers in Spanish or Amharic and only use English to converse with administrators and teachers. Most of the ELL students in my classes have sufficient command of English and require only limited support, but there are a handful who may require substantial support (e.g. dual-language academic resources, translators, tutors, etc.).

Recent standardized test scores suggest significant deficits in English Language Arts, math, and science performance. Student surveys and anecdotal observations reflect a disinclination toward the sciences among the student population. Often the students' lack of interest in the sciences is a substantial factor in lack of classroom engagement. An expectation for this curriculum unit is to increase classroom engagement by emphasizing the direct connection between the textbook/academic material that students might find abstract or even arbitrary ("*Why do we have to learn this?*") and the contemporary real-world applications of those concepts. The hope is that students will embrace the idea that the foundational principles being introduced in class are the basis for new, life-altering medical advances impacting the lives of real people. This is a version of a notion I introduce on the first day of school and underscore throughout the year with each new unit; in academia in general, but in science more particularly, the point of learning is not simply to come to know information that others have previously discovered or described, but rather to acquire skills and insight that will lead you to develop new knowledge to be shared with and advance the world.

Standards

The curriculum unit is intended for 11th and 12th grade Anatomy and Physiology students who may or may not have previously completed a high school biology course, and still lack mastery of some foundational concepts from biology. The curriculum unit incorporates Next Generation Science Standards (NGSS) for High School Life Sciences (HS-LS1: From Molecules to Organisms: Structures and Processes and HS-LS3: Heredity: Inheritance and Variation of Traits) and specifically emphasize the associated Disciplinary Core Ideas (DCI) (LS1.A: Structure and Function, LS1.B: Growth and Development of Organisms, LS3.A: Inheritance of Traits, and LS3.B: Variation of Traits).

The performance expectations in HS-LS1 are intended to help students formulate an answer to the question, *"How do organisms live and grow?"* The content of the curriculum unit addresses whole-body effects of the skeletal system and the roles it plays in sustaining the human organism (i.e. the primary functions of the skeletal system presented previously) as well as the requirements needed to maintain life at the level of the

cell. Because many approaches for augmenting bone regeneration aim to enhance cellular and sub-cellular activities, students are able to draw a direct line from the coordinated activity and inactivity of different cells or organelles (e.g. osteoblasts and osteoclasts) and the specific roles they play (building or removing bone components) through to the organ system (skeleton) and the variety of functions they perform (e.g. providing support and protection).

The performance expectations in HS-LS3 are intended to help students formulate answers to the questions, *"How are characteristics of one generation passed to the next? How can individuals of the same species and even siblings have different characteristics?"* Examining approaches for augmenting bone regeneration that may involve manipulating DNA provides the avenue to address genetics and heredity in this unit.

The curriculum unit necessarily also incorporates NGSS Science & Engineering Practices (SEP); 1 - asking questions (science) and defining problems (engineering), 2 - developing and using models, 3 - planning and carrying out investigations, 4 - analyzing and interpreting data, 5 - using mathematics and computational thinking, 6 - constructing explanations (science) and designing solutions (engineering), 7 - engaging in argument from evidence, 8 - obtaining, evaluating, and communicating information.

The Curriculum Unit

The curriculum unit is arranged in three parts. Part one presents an overview of the processes and mechanisms of normal bone development and remodeling. There is a specific focus on the roles of the various cell types and cellular components. Part two is an examination of established, novel, theoretical restorative therapies for bone and joint conditions. Part three is an exploration of the risks, consequences, and ethical considerations related to various restorative therapies for bone and joint conditions.

Part One - Overview of the processes and mechanisms of normal bone development and remodeling

Previous parts of the larger skeletal system unit will have introduced students to the various processes that result in the bones that support their bodies and allow them to move among other functions. In short, before engaging with part one of the curriculum unit, students will understand that bone formation begins early in fetal development and includes intramembranous ossification (the osteogenic process by which the clavicles and most of the skull form) and endochondral ossification (the osteogenic process by which long bones like the humerus and irregular bones like those comprising the vertebral column and pelvis are formed) (Shier, Butler, & Lewis, 2013).

Students readily observe and embrace the idea that through infancy, childhood, and adolescence bones grow in size and strength; that much is plain to see. What is less obvious to students, is that adult bones are not inactive, inanimate objects waiting to be acted upon by muscles or other organs. The bones of an adult, indeed all bones, are dynamic living tissues that are constantly undergoing changes in response to environmental and/or mechanical stressors (Shier, Butler, & Lewis, 2013).

Bone remodeling is an essential process for maintaining bone strength and mineral homeostasis (Shier, Butler, & Lewis, 2013). Bone remodeling allows the body to repair old damaged bone and make structural adjustments in response to the loads the bone must support. The process on bone remodeling is intricate and

coordinated yet evokes a certain simplistic elegance when considered as a whole. Understanding this process and the roles played by the specialized bone cells involved (osteocytes, osteoclasts, and osteoblasts) is critical to begin answering the question *"How can humans take advantage and manipulate bone physiology to enhance bone-related medical interventions?"*

The following imperfect yet effective analogy will allow students to grasp the phases of the bone remodeling cycle (*activation, resorption, reversal, formation, quiescence*). Bone remodeling is akin to the process of remodeling a worn damaged house and the various people involved can be loosely analogized to the *osteocytes*-mature bone cells, *osteoclasts*-bone removers, and *osteoblasts*-bone builders). To begin, *phase 1 – activation*, the home owner must signal to the construction company that the building is in disrepair and in need of remodeling. In this way the homeowner is not unlike an osteocyte. Osteocytes are fully differentiated osteoblasts trapped within the bone matrix. In response to stresses, for example a mechanical load on the bone, osteocytes in the region of the load respond by releasing signal factors. It is through these signal factors, that osteocytes regulate osteoclast and osteoblast activity and thus coordinate bone remodeling (Knothe Tate, Adamson, Tami, & Bauer, 2004). As the process of remodeling a home involves an initial crew with particular tools for stripping away much of the old structure, so does *phase 2 – resorption*, involve removing the old damaged bone. In this phase osteoclasts employ acids and enzymes to remove the organic and mineral components of bone (Knothe Tate, Adamson, Tami, & Bauer, 2004). It is only after the old parts are removed that there can be a shift in process. This is comparable to *phase 3 – reversal*, in which the osteoclasts leave the remodeling site. In *phase 4 – formation*, a new crew with different tools arrives to begin the new construction. Osteoblasts secrete a collagen matrix in the resorption pit and control its mineralization forming new bone (Knothe Tate, Adamson, Tami, & Bauer, 2004). After formation the system rests in *phase 5 – quiescence*, until such a time as there is another signal for activation. When students are able to identify factors in the construction scenario that might improve or speed-up results (e.g. more or more active construction workers, more or better tools, more or better raw materials), so too can students begin to contextualize the roles of growth factors like bone morphogenic proteins.

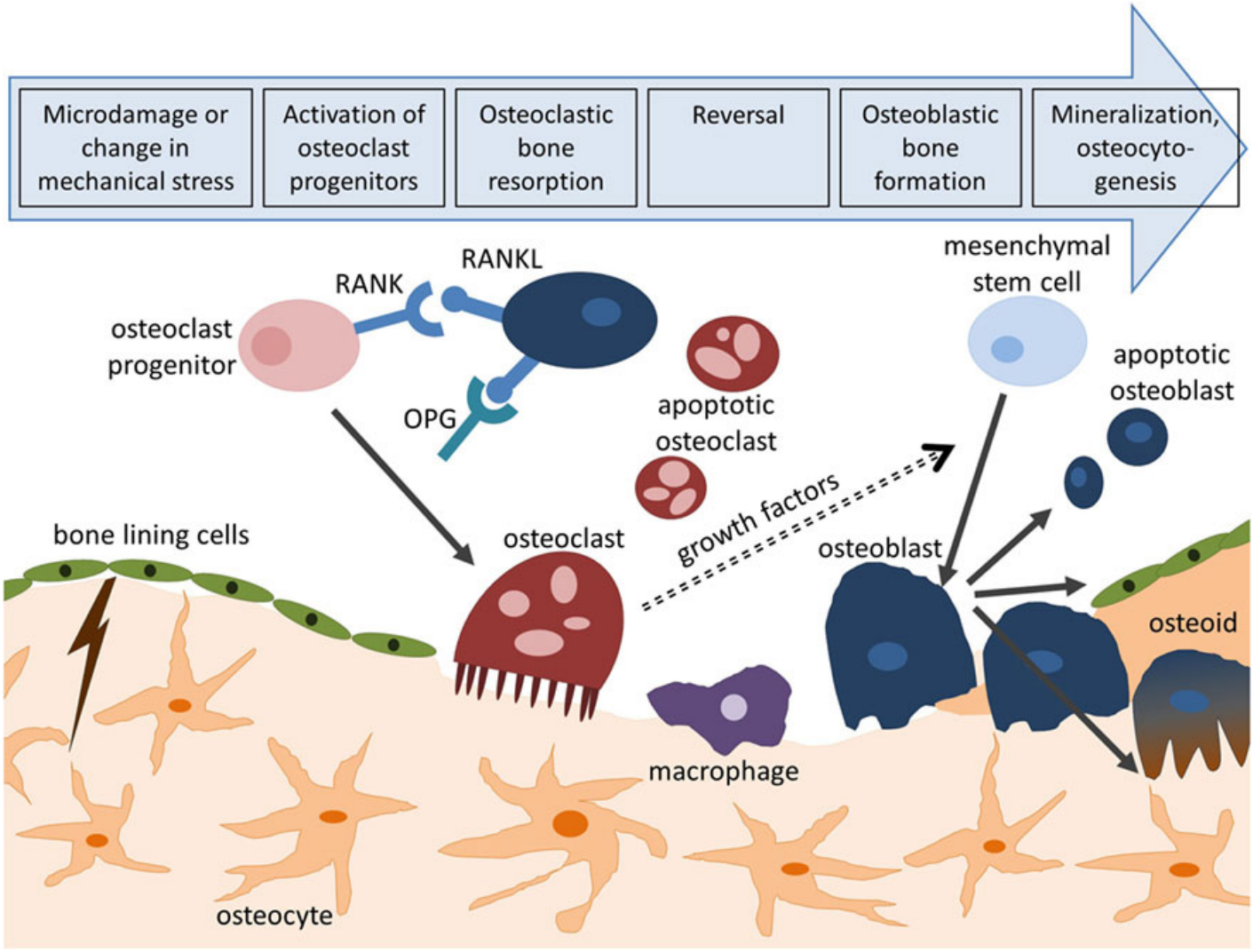

Figure 1 - Bone Remodeling Cycle

Part Two – Augmenting Bone Regeneration: Restorative Therapies (Established, Novel, and Theoretical)

Both in the United States and worldwide, the need for effective and efficient restorative therapies for bone loss is substantial. Bone loss or damage due to musculoskeletal disease or injury has significant medical and economic ramifications on individuals and society more broadly. The American Academy of Orthopedic Surgeons periodically catalogues these impacts in *The Burden of the Musculoskeletal Diseases in the United States: Prevalence, Societal, and Economic Cost*, most recently in 2014. Key takeaways from the most recent volume of *Burden* included the following: musculoskeletal conditions are associated with nearly 600 million physician visits, 400 million visits to non-physician health providers, as many home health care visits, and more than 20 million hospital admissions. In the most recently documented 2-year period, persons with musculoskeletal conditions averaged nearly 21 prescriptions per person filled for these conditions, totaling more than 2 billion prescriptions. In the same period, the aggregate economic impact of musculoskeletal conditions was near $800 billion (Initiative, 2014). Worldwide, osteoporosis alone accounts for 9 million fractures annually; an average of 1 fracture every 3 seconds (International Osteoporosis Foundation, 2017). Data suggest that as much as 10 percent of fractures fail to heal properly (R.T. Franceschi, 2005). In this context, the critical need for safe, effective restorative therapies to stimulate bone regeneration is self-

evident.

The primary goal for this section of the curriculum unit is for students to engage with and reinforce the fundamental idea that health ailments are just changes in functional capability owing to changes in structure. Students will stress the idea that solutions for bodily maladies arise from restoring normal structure and/or mollifying the effects of abnormal structure; once again *structure-function-dysfunction.* In analyzing each of the restorative therapies to be presented in this section and comparing between them, students will draw on their understanding of normal bone development and remodeling (presented in part one). The therapies are presented as real-world concrete applications of content students are mastering using a simple, but logical and coherent framework. The details (mechanisms of action, procedures, and the like) are important, but in teaching this curriculum unit, the points of emphasis will be on the overarching idea of how the big ideas fit together. Additionally, the particular methods presented in the subsections to follow are not meant to represent an exhaustive list of medical interventions in conditions of bone loss or bone damage. Rather, they are compelling examples chosen in furtherance of illustrating the big picture. Other instructors, or indeed the author in subsequent offerings of the course, could very well substitute other interventions while still allowing the curriculum unit to achieve its overall aim.

Bone Grafting

Bone grafting is the go-to procedure for bone reconstruction and fracture healing in cases of surgery, trauma, and degeneration as have been described. (Lu, Chang, Lin, Li, & Hu, 2013) More than 500,000 of these procedures are performed each year in the U.S.; globally the estimate more than doubles. (Greenwald, et al., 2001) Bone grafting is a surgical procedure that replaces missing bone to allow for the appropriate spacing and/or scaffolding required for the biological processes involved in regenerating bones – osteogenesis, osteoconduction, and osteoinduction. Osteogenesis involves the formation of new bone from transplantation of osteocompetent cells (i.e. osteoblasts). Osteoinduction involves formation of new bone from the differentiation and stimulation of undifferentiated mesenchymal stem cells. Osteoconduction involves formation of new bone along a scaffold from the host osteocompetent cells at the recipient site. (Roberts & Rosenbaum, 2012)

There are various types of bone grafts differentiated largely based on the source of the transplanted bone - autologous bone grafts, allogeneic bone grafts, xerographs and synthetic bone substitutes. Each variation has particular risks and benefits associated, as does the general procedure itself. Autologous bone grafts involve harvesting donor bone from sites of non-essential bone (e.g. the iliac crest of the pelvis) in the same individual receiving the graft. Autologous grafts are the gold standard because the transplanted bone possesses all the necessary biological processes. Furthermore, these grafts are by nature immunocompatibile, thus bypassing risks of rejection and disease transmission. Still, there are drawbacks. Autologous grafts may be limited by the amount of bone available from the donor site, donor site morbidity, and the need for bone harvesting procedures (surgical risk at a second site) (Lu, Chang, Lin, Li, & Hu, 2013). Allografts are obtained from individuals of the same species, typically cadavers, thus alleviating donor site concerns associated with autographs. The tradeoff is an increased risk of infection and disease transmission. Alloplastic (synthetic) graft alternatives lack bioactive properties and thus can only serve in an osteoconductive capacity (Lu, Chang, Lin, Li, & Hu, 2013).

Augmentation with protein growth factors

Of note in the previous section is that the interventions described were relatively simple in terms of the overall scheme; surgeons merely sought to enhance endogenous regeneration processes by inserting

structures that approximate the normal state. Even when the interventions appear to increase in complexity, there is an adherence to such a principle. This is evident with restorative therapies involving growth factors. The application of biological signaling molecules (growth factors in this case) seek to stimulate the host's natural healing responses and regenerative repair capabilities while avoiding the disadvantages of the more invasive procedures described in the previous section.

An apt example for this sort of intervention is application of born morphogenic proteins (BMPs), the most well-studied growth factors in bone regeneration. The BMPs are a large group of structurally related proteins that belong to the transforming growth factor-beta (TGF-β) superfamily. By mechanisms of action involving signaling the chemotaxis, proliferation, and differentiation of osteoprogenitor cells, and ultimately, the induction of bone formation by these cells, multiple BMPs have been shown to be closely involved in the processes of bone formation and regeneration (Nauth, Ristevski, Li, & Schemitsch, 2011). For several decades researchers have been able to use restriction enzymes to insert genes from one organism (e.g. human BMP) into the genome of another (e.g. a *Escherichia coli* plasmid) – recombinant DNA. In such an example, the bacterial mechanism could be highjacked to produce a human protein. By these recombinant methods, two BMPs are widely available and approved for limited clinical use; recombinant human (rh)BMP-2 and rhBMP-7 (Nauth, Ristevski, Li, & Schemitsch, 2011). The effectiveness of these bacteria-produced human protein products in promoting bone regeneration has been evaluated in clinical studies of non-union, bone defects, open tibial fractures, and spinal fusion, with some trials showing regeneration results comparable to auto graft procedures. One such study (Friedlaender, et al., 2001) examined tibial non-union patients randomized in two groups: one group received autologous bone grafts, the other received rhBMP-7. All other aspects of their restorative therapy were identical. The data from the Friedlaender group showed the two groups to be comparable in ability to support weight (81% of the recombinant cohort versus 85% of the graft cohort) and radiographic evidence of union (75% versus 84% respectively). The rate of infection in the recombinant cohort was a third of that of the graft group (4.9 % versus 13%). Furthermore, by definition none of the recombinant cohort, experienced the long-term persistent donor site pain reported by 20% of the graft cohort. In sum, the Friedlaender data showed equivalent results for BMP-based therapy when compared to autologous grafting, while avoiding the morbidity of graft harvest (Friedlaender, et al., 2001).

While the advantages of protein growth factors are significant and promising, there are limitations that make their use in many cases less than ideal. Human proteins produced by *E. coli* bacterial machinery are not modified in the same way as by human cells. Recombinant proteins of this kind lack the post-translational glycosylation (modifications made after initial protein assemble for proper folding, stability or other purposes) that is present in the endogenous protein (Lo, Ashe, Kan, & Laurencin, 2012). Consequently, these recombinant growth factors tend to be less stable and less biologically active. In part due to the limited bioactivity, treatments of this kind require high doses (which are given by injection) (Southwood, Acvs, Frisbie, Kawcak, & Wayne Mcilwraith, 2004). It is speculated that *E. coli*'s central role in recombinant growth factor production also increases the possibility of contamination from trace amounts of biologically active impurities. Owing to their relative size (compared to non-protein substances) these protein growth factors are more likely to induce unwanted immune responses from the host. Beyond these drawbacks of the recombinant protein product, the cost of the manufacturing process itself is significant (Southwood, Acvs, Frisbie, Kawcak, & Wayne Mcilwraith, 2004). The burden of high cost to produce a large molecule with some instability and the potential to trigger an immune response currently limit the utility of this restorative therapy .

Augmentation with small molecules

In the previous subsection, the limitations on the effectiveness of the interventions was in large part

associated with the size of the protein growth factors. Comparatively, even small proteins are big in terms of biological molecules. In this context then, a substance with the bone-promoting properties of growth factors but without the concomitant heft would be of great interest to researchers; possibly delivering the benefits without many of the drawbacks. And indeed, research does seem to show that such molecules counteract each of the previously described drawbacks. With regard to immunogenicity, small molecules can be small enough to avoid triggering an immune response. Considering stability, whereas proteins generally need to be stable and maintain particular orientations to retain bioactivity, small molecules do not require the similar levels of structural integrity and rigidity (Lo, Ashe, Kan, & Laurencin, 2012). Not insignificantly, large protein growth factors tend to require high doses and are generally administered via injections (editorial note: *multiple* injections, probably with big needles – because for those who are averse to needles, is there really a such thing as a "not big" needle?), whereas smaller molecules are more likely to be administered orally (Lo, Ashe, Kan, & Laurencin, 2012). And lastly, compared to the process of manufacturing recombinant proteins, many small molecule drugs are inexpensive organic compounds (Lo, Ashe, Kan, & Laurencin, 2012).

Of course, these molecules also have their own drawbacks, namely non-specificity and shorter half-lives. Even still, the discovery of small molecules with osteoblastic differentiation capability is of great interest to researchers in this field. Molecules of this class include but are certainly not limited to: purmorphamine, statins, phenamil, rapamycin, and icariin among several others. (Lo, Ashe, Kan, & Laurencin, 2012) Any of these would probably be appropriate as the example for this subsection of the curriculum unit, but an even more intriguing example takes the idea to an extreme of sorts; even smaller molecules – ions. Several ions - boron (B^{3+}), calcium (Ca^{2+}), cobalt (Co^{2+}), copper(II) (Cu^{2+}), fluoride (F^{-}), lithium (Li^{+}), magnesium (Mg^{2+}), niobium (Nb^{5+}), phosphate (PO_4^{3-}), silicate (Si^{4-}), silver (Ag^{+}), strontium (Sr^{2+}), vanadium (V^{5+}), and zinc (Zn^{2+}) - have been shown to be capable of inducing osteoblast precursor differentiation through growth factor signaling pathways, or to stimulate other processes in support of bone tissue growth (Lo, Ashe, Kan, & Laurencin, 2012). The size-related benefits (lower cost, stability, efficacy at low concentrations, etc.) highlighted previously pertain here as well. A further distinction is that these molecules are even simpler than the other molecules. Non-specificity and shortened half-lives are still drawbacks, and the challenge for research is to develop techniques for delivery-appropriate dosages in targeted locations over time. Still, such challenges should be more manageable with these molecules in comparison to larger, more unwieldy substances.

Augmentation by Gene Therapy

The progression of the preceding subsections moves toward interventions on smaller and smaller scales. Though this progression lends a sort of logical flow to the curriculum unit, it is certainly not intended to suggest that simply reducing the physical scale of the intervention inevitably makes for an improved or even preferred outcome. It is the precision of the intervention that matters, not necessarily the size of mediating structures involved. The interventions presented in this section will provide examples of targeted precision by another means; namely, gene therapy.

One of the significant limitations of protein growth factor mediated bone regeneration is the reduced stability and bioactivity of the recombinant protein due to a lack of glycosylation resulting from the use of *E. coli* cellular machinery. These less-stable, less-active recombinant proteins are then more likely to degrade too soon and less effectively promote bone formation. Gene therapeutic approaches are able to avoid such challenges because rather than transferring a large *E. coli*-made protein, vectors are used to transfer the genes. In this way, the hosts cellular machinery is used to produce a protein the undergoes the appropriate post-translational processing (e.g. glycosylation). Such a protein, with improved stability and bioactivity will

more effectively function to help to regenerate bone.

After having examined these previous established and some novel approaches for augmenting bone regeneration, students will conclude this part of the unit by considering interventions that may be on the horizon. Perhaps no scientific development holds more potential for advancing medical intervention than gene editing approaches using CRISPR-based technology. The advent of CRISPR technology has had wide reaching impact in many areas of medical science and in the wider public imagination. Researchers are beginning to view bone regeneration as one such area of potential gene-based interventions. Here, students will be introduced to the basic mechanisms of the CRISPR machinery and be presented with non-bone examples of CRISPR applications, so that they can consider potential bone-regeneration applications.

So, what exactly is CRISPR? In short, CRISPR technology is a simple yet powerful tool that allows for editing of an organism's genome. CRISPR (or Clustered Regularly Interspaced Short Palindromic Repeats) is an evolved defense mechanism that naturally occurs in some species of bacteria (Cong & Zhang, 2015). CRISPR provides a defense for cells by readily identifying invading viruses based on fragments of viral DNA from previous attacks that have been incorporated in a CRISPR array in the bacterial chromosome. Together with the bacterial protein Cas9, the system is able to cut and remove parts of the virus in order to deactivate it. Researchers have taken advantage of this bacterial defense mechanism and designed a CRISPR-Cas9 system that can target and edit DNA at precise locations [See figure 2]. A cell is transfected with a DNA plasmid that expresses both the Cas9 protein and a sequence of guide RNA (gRNA), which matches and binds to the gene of interest. The Cas9 protein then identifies the corresponding sequence on the host cell's genome and initiates a double stranded cut in the DNA. The double strand cuts activate a cascade of signals whose ultimate goal is to repair the cuts, which will occur in one of two ways (3a and 3b in figure 2). By one method, non-homologous end joining (NHEJ), the targeted gene is removed because cut ends of DNA are joined together without the gene between them. In the other method, homology directed repair (HDR), a replacement gene whose flanking sequences were designed to match the cut sites is inserted in place of the original gene (Cong & Zhang, 2015).

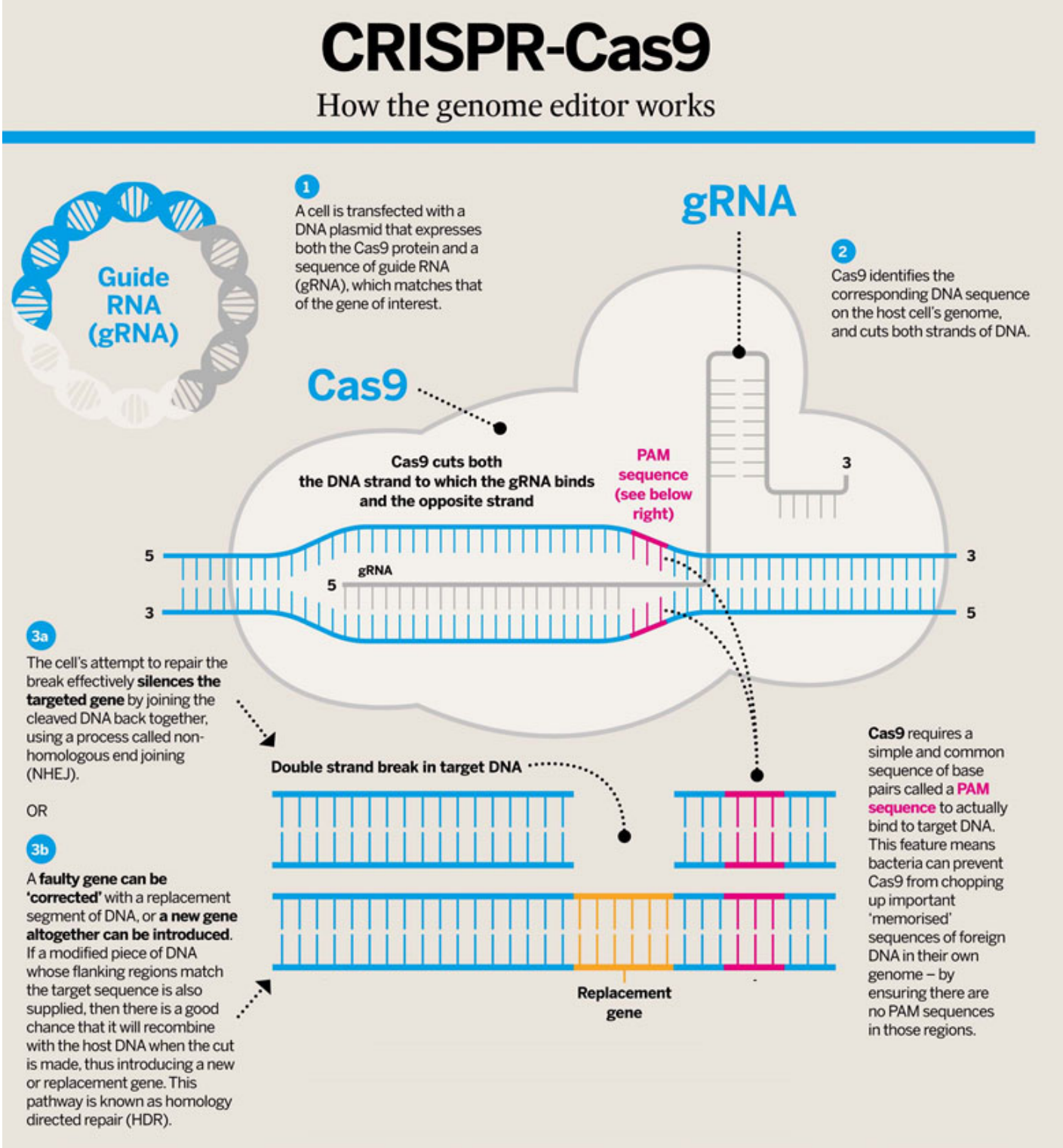

Figure 2- CRISPR-Cas9 Mechanisms

With such a tool, the gene therapeutic possibilities are vast. One such example of the applications of CRISPR comes from the Parker Institute for Cancer Immunotherapy. Using the CRISPR-Cas9 system the researchers designed an elegant approach to help the body fight cancer. In this novel approach, researchers remove T cells from cancer patients and perform three CRISPR edits on them. One edit inserts a gene for a protein engineered to detect cancer cells and instructs the T cells to target them. Another edit deletes a gene for an endogenous T cell protein, PD-1, that normally inhibits T cell response (a check point of sorts). The last edit

inhibits cancer cells to disable T cells because it removes a protein that identifies the T cells as immune cells. The modified T cells are then returned to the patient (Reardon, 2016) (Engineering T-Cells to Fight Cancer | Parker Institute for Cancer Immunotherapy, 2017). The simplicity and specificity of the CRISPR allows these scientists to construct a two-pronged approach that both enhances the ability to attack cancer cells and inhibits the ability of the cancer cells to attack the T cells. Early results of the study are promising, and the approach has already been approved for human clinical trials.

Presently the technology is so new, that little research about the possible bone regeneration gene targets of CRISPR technology have been published. This situation presents a unique opportunity to challenge students to develop their own gene therapeutic CRISPR-mediated schemes that could be predicted to have positive outcomes. The big picture construction of CRISPR technology (*it's as easy as removing a typo from a sentence and re-writing it correctly*) can be easily understood by students and as such there should be some overlap in schemes suggested by students and the work that researchers are pursuing. For example, following the T cell example in the previous paragraph, a student with even a basic understanding of bone regeneration and of CRISPR technology could think some version of the following: "my *goal is to have bone heal and regenerate itself more quickly and more efficiently. What if I delete genes that slow down repair and spread genes that speed up repair?"*

In a recent abstract for a research proposal, Dr. Adalberto Luiz Rosa wrote that he has selected genes involved in either promotion or inhibition of osteogenesis as targets in a CRISPR-based approach to augmenting bone regeneration. His group intends to use CRISPR to overexpress *BMP*-9 which initiates cartilage and bone formation and also knock out periodontal ligament-associated protein-1 (PLAP-1) which acts as an inhibitory factor for osteogenic differentiation and bone formation. Rosa expects data from this research will lead to viable alternative therapies for the repair of bone defects and non-union fractures (Rosa, 2017). The simplicity of the approach and its relation to the very questions students were expected to have generated earlier, allows this research proposal to serve as another point of emphasis for connection between basic principles explored in the classroom and cutting-edge medical interventions being developed in the real world.

Part 3 – Scientific Judgement: Risks, Consequences and Ethical Considerations of research.

CRISPR technology and its potential have captured both the scientific and popular imaginations. Researchers and laypeople alike are intrigued and absorbed by its seemingly endless capacity to provide a remedy for every condition from cancers to male pattern baldness. In this exuberant context and the drive to push the possibilities forward, there is the argument that perhaps insufficient consideration is given to risks and dilemmas arising from the new and advancing capabilities. In this part of curriculum unit students will consider risks and consequences associated with gene-based therapies, and then grapple with some of the ethical conundrums newly emphasized in the development of this technology.

The pace at which CRISPR technology has spread is remarkable. In little more than a half-decade since its emergence, it continues to gain momentum in research and laboratory settings because it is considered precise, fast, cheap, and easy to use. It is also for these very reasons that is necessary to pause and soberly assess the risks. In a 2017 review published in the Yale Journal of Biology and Medicine, Cribbs and Perera summarized some of the most compelling potential risks associated with CRISPR-Cas9 gene editing technology. Cribbs and Perera divided potential risk into two groups; technical concerns and social concerns. Within the technical group were further subdivision for off-target insertions and deletions (Indels), random integration of vector, and toxicity. Across all categories risks were considered for germline editing, ex vivo delivery (cell transplants), and in vivo delivery (tissues and organs) (Cribbs & Perera, 2017). The results are

summarized in table 1.

Table 1: The potential risks associated with CRISPR-Cas9 gene editing technology.

Specific CRISPR-Cas9-based applications in humans: Potential risks

	Technical			Social
	Off-target insertions and deletions (Indels)	Random integration of vector	Toxicity	Exacerbating social inequalities
Germline editing	**High** - A potentially significant issue but screening of embryos prior to implantation could overcome this risk.	**Medium** - Random integration may result in inactivation/dysregulation of gene expression. Sequencing for the presence of integration would identify this.	Low	**High** – Dependency upon "enhancement" applications
Ex vivo delivery (cell transplants)	**High** - For clonally expanded cells, screening can be performed to identify off-target Indels.	Low	Low	Low
In vivo delivery (tissues & organs)	**High** - Poses the most significant risk because screening would be difficult to perform.	**High** – It would be very difficult to determine integration in vivo. If the integration occurs in a tumor suppressor or oncogene the development of cancer could be an issue.	**High** – The induction of inflammatory responses to vector components.	Low

One take-away apparent from the table is the relative high risk associated with potential inadvertent insertions and deletions (indels). That is the example to be focused on in this part of the unit. In downplaying the possibility of unintended and unforeseen consequences, researchers have lauded the specificity of CRISPR targeting; CRISPR is specific because the gRNA pairs with a unique sequence to initiate the mechanism. There has been a general acceptance of these conclusions about specificity because to this point exploration of Cas9-induced genetic alterations has been limited to the immediate vicinity of the target site and distal off-target sequences (Kosicki, Tomberg, & Bradley, 2018). However, in a July 2018 paper in Nature, researchers from the Wellcome Sanger Institute call for more scrutiny and caution in using CRISPR technologies, citing more unintended mutagenic events associated with CRISPR than previously reported. Whereas the majority of DNA repair outcomes in CRISPR were thought to be indels of less than 20bp, they report large deletions of thousands of base pairs and other complex rearrangements (Kosicki, Tomberg, & Bradley, 2018). The WSI researchers speculated that a substantial portion of genotypes resulting from Cas9 repair may have been missed for several reasons (e.g. the research often used cancer cells with abnormal DNA repair mechanism thus making it problematic to extrapolate to normal cells – also the scope of the assessment was limited because studies relied on amplification of short regions around the target and potential off-target sites.) A significant concern is that, in actively reproducing cells, these newly reported deletions and rearrangements can have significant pathogenic consequences (e.g. cancer) (Cribbs & Perera, 2017).

These results emphasize the need for deliberate caution as appropriate when pushing forward with CRISPR technology. Beyond the obvious concern for individual patient outcomes, it is also important to note that with regard to research in medical science, unintended consequences that lead to bad individual outcomes, can resonate across the field and set back all manner of promising research (especially when they play in to existing public skepticism and unease).

Beyond questions of efficacy and safety, gene therapies are often controversial due to associated moral and ethical questions. As with other scientific developments, as the technology advances, questions shift from 'can we', to 'should we'.

By this part of the curriculum unit, students now have a firmer foundation of science on which they can meaningful engage with some of the pertinent ethical questions of the day.

Among other issues, students will consider the following questions presented on the NIH Genetics Home Reference website: *How can "good" and "bad" uses of gene therapy be distinguished? Who decides which traits are normal and which constitute a disability or disorder? Will the high costs of gene therapy make it available only to the wealthy? Could the widespread use of gene therapy make society less accepting of people who are different? Should people be allowed to use gene therapy to enhance basic human traits such as height, intelligence, or athletic ability?*

Ultimately students should be about to weigh risks and benefits (present day and potential) associated the gene-based therapies (CRISPR in particular) and formulate responses to the question "*When do the benefits outweigh the risks?"*

Classroom Activities

Research Proposal and Academic Conference Poster

Students will outline some detailed research plans that, if successful, would advance the field of augmenting bone regeneration. The most important elements to include in such a proposal are the rationale for the research, the particular structures and functions to be explored by the research, and the methods of inquiry, and data analysis to be used (where possible). Students will have to develop at least two different plans: one that is to incorporate a CRISPR-Cas9 technique and another that does not. It will be emphasized that the methods presented in this unit were presented as examples and not intended to be an exhaustive list. Students will be encouraged to research methods of bone regeneration beyond those examples specifically presented here. Students will then make an academic conference-style poster based on their research proposal. The "First Annual Roosevelt High School Bone Regeneration Research Symposium" will be held at the close of the unit. Where possible, other science teachers will bring their students to attend the symposium and ask questions of presenters. A conference committee comprising science and other teachers will convene to select symposium award recipients, in such categories as most innovative proposal and most well-presented proposal.

Fixing Mr. Glass and David Dunn

In the movie *Unbreakable* (released in 2000, directed by M. Night Shyamalan) two of the characters have unique bone anatomy and physiology. One character, Mr. Glass played by Samuel L. Jackson, has extremely fragile bones. The other, David Dunn played by Bruce Willis, incredibly resilient physical features that seem indeed unbreakable. The assignment will involve students drawing on their understandings of various methods of bone augmentation, to develop a set of procedures that might be able to be used to "fix" the boney conditions of those two characters.

Data Analysis and Conclusions

One emphasis of this unit is analyzing and interpreting data, one of the important NGSS Science & Engineering Practices. In this learning activity students are provided data sets, tables, graphs, etc. from published research journal articles about bone regeneration. Students then are asked to examine the data and use a graphic organizer to make sense of the information presented. The graphic organizer is simple and contains sets of three boxes, each with a set of sentences intended to guide the student's exploration of the

data.

Box 1 questions - Describe the data (*what kind of information, how is it organized, where did it come from, etc.*). Box 2 questions - What *patterns/trends/correlations* do you notice in these data? Which trends in the data stand out the most? Do all the data fit these patterns (*Are there any outliers? Are there unique elements/features of the data*)? Box 3 questions - What do the patterns/trends/correlations lead you to *conclude* (*What might the trends and patterns mean*)? (See Figure 3)

When students analyze multiple pieces of data from the same research or related content, they then can use a more expansive version of this graphic organizer to craft a clear scientific argument that is founded in data and data analysis. The information presented in the conclusion boxes (box 3 for each piece of data) can be combined to make an evidenced-based claim that is the foundation of a sound argument.

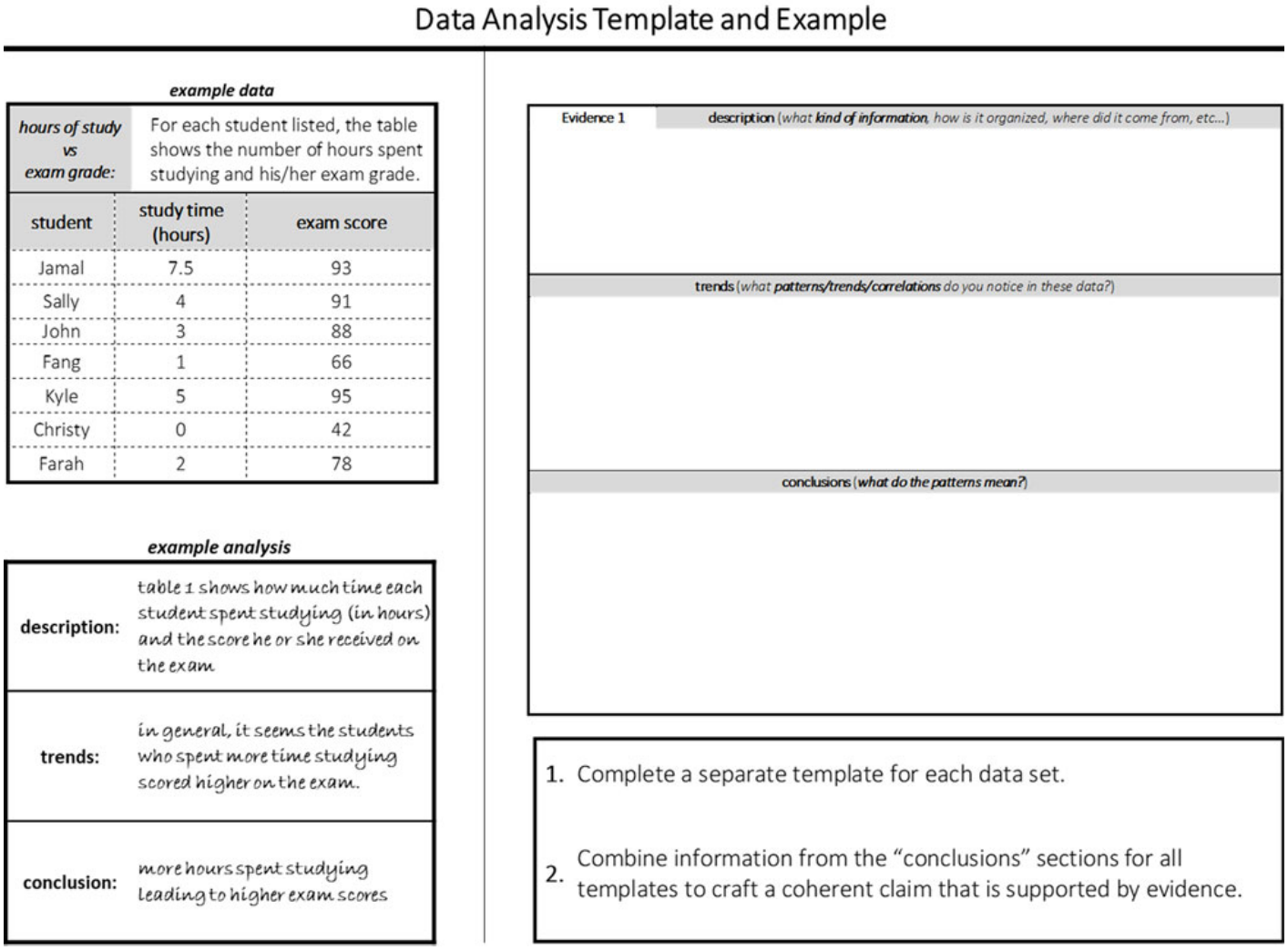

Figure 3 - Data Analysis Template and Example

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