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Regulation of regeneration by Heparan Sulfate Proteoglycans in the Extracellular Matrix

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Abstract

Just as the building of a house requires a blueprint, the rebuilding of lost or damaged body parts through regeneration requires a set of instructions for the assembly of the various tissues into the right places. Much progress has been made in understanding how to control the differentiation of different cell types to provide the building blocks for regeneration, such as bone, muscle, blood vessels and nerves/Schwann cells. These are the cells that follow the blueprint (the pattern-following cells) and end up in the right places relative to each other in order to restore the lost function. Much less is known about the cells that are specialized to generate and regenerate the blueprint (the pattern-forming cells) in order to instruct the pattern-following cells as to how and where to rebuild the structures. Recent studies provide evidence that the pattern-forming cells synthesize an information-rich extracellular matrix (ECM) that controls the behavior of pattern-following cells leading to the regeneration of limb structures. The ability of the ECM to do this is associated with glycosaminoglycans that have specific spatial and temporal modifications of sulfation patterns. This mechanism for controlling pattern formation appears to be conserved between salamanders and mammals, and thus the next challenge for inducing human regeneration is to identify and understand the biology of these pattern-forming cells and the ECM that they synthesize.

Keywords

axolotl; regeneration; regenerative engineering; growth factor; heparan sulfate; ECM

Introduction

Just as the building of a house requires a blueprint, the rebuilding of lost or damaged body parts through regeneration requires a set of instructions for the assembly of the various tissues into the right places. In the case of the house, the blueprint ensures that the plumbing for the toilet ends up in the bathroom and not in the kitchen; in the case of regeneration (e.g. a limb), it is necessary that the arrangement of bones, muscles and nerves is correct in order

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Compliance with Ethical Standards

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for the limb to function. Therefore, in order to achieve the ultimate goal of inducing human regeneration, it will be necessary to discover how to remake the blueprint in addition to recruiting the cells to remake the various tissues.

We know that there is a blueprint for regeneration based on studies from model organisms, such as salamanders, that can regenerate. This has been demonstrated in classic experiments in which tissues are grafted from one location to another so as to bring cells together that normally would not interact with each other. In response to these new interactions, growth is stimulated and extra (supernumerary) structures (e.g. extra limbs) are induced to form [1,2]. Such experiments demonstrate that the interacting cells have information about their normal location within the limb (referred to as positional information), and that they can recognize the positional information on adjacent cells. Thus when grafted to a new location, these cells interact with host cells with different positional information, which stimulates growth and supernumerary limb pattern; a process referred to as intercalation ([1,2] see below).

The cells with the ability to differentiate into the various limb tissues are equivalent to the building blocks for making a new house (e.g. bricks, lumber, pipes and wires). For many tissues, there are adult stem cells that can be recruited to regenerate tissues in a lineage-restricted manner (e.g. muscle cells derived from muscle associated satellite cells). Progenitor cells for other tissues can be derived from differentiated cells that can be induced to dedifferentiate using techniques for generating induce pluripotent cells (iPSCs), or through undifferentiated, multipotent stem cells (e.g. embryonic stem cells). These undifferentiated cells can then be led along well-characterized developmental pathways leading to the differentiation of specific cell types. The ever increasing rate of discoveries in developmental genetics and stem cell biology has either already achieved, or soon with lead to the ability to recruit the necessary numbers of cells, and to control their fate inorder to provide the building blocks for regeneration.

Looking forward, it appears that the challenge for inducing human regeneration will be to provide the positional information blueprint for the regeneration-competent cells. At this point, little is known about the biochemical properties of positional information; however, there is one possible strategy for controlling it. Sulfated glycosaminoglycans [heparan sulfate proteoglycan (HSPG) in particular] can control the spatial and temporal activities of growth factor/morphogen signaling (e.g. FGF and BMP) via specific modifications in the patterns of sulfation [3,4]. These are the signaling molecules that function during embryonic and regenerative development to control growth and pattern formation (see [5]), and thus controlling the patterns of HSPG sulfation would allow for the spatial and temporal regulation of growth factor/morphogen signaling. Consistent with this hypothesis is the report that HSPGs in the ECM of both axolotl and mouse limb skin can induce pattern formation in regenerating limb blastemas in the axolotl [6]. Similarly, regulation of growth and pattern formation by spatial and temporal modifications of heparan sulfates has been hypothesized for developing chick limb buds [7,8], and for Xenopus limbs during development and regeneration [9]. Collectively, these findings implicate heparan sulfate modifications as being involved in positional signaling that would correspond to positional information in mouse, chick, Xenopus, and axolotl ECM.

As a first principle, it is intuitive that positional information is a property of a subpopulation of cells within the limb (or any other organ), and thus an initial challenge is to identify those cells. Although the identity of the blueprint cells has not yet been discovered, it is known that they are located within the loose connective tissue compartment of the limb [1,2,10,11]. Thus there are cells are specialized to generate and regenerate the blueprint (the pattern-forming cells), and there are cells that are specialized to build and rebuild the structures (the pattern-following cells), such as bone, muscle, blood vessels and nerves/Schwann cells [10]. The pattern-forming cells instruct all the other cells as to where to go, and thus the next challenge for inducing human regeneration is to identify and understand the biology of these cells.

Positional information: the blueprint for regeneration

The evidence for cells with different positional information (i.e. the pattern formation cells) comes from experiments in which tissues (e.g. skin) are grafted from one location to another resulting in the stimulation of extra growth and pattern (intercalation). The principles associated with this phenomenon were formalized in the Polar Coordinate Model [1,2,12], in which cells have information of their location within structures, much like a street address and postal zip code.

The cells reference their positional information when they interact with each other such that cells with the same code are normally neighbors, and intercalation is not stimulated. This normal positional relationship can be altered experimentally by grafting limb tissue from one location to another (e.g. cells from the left side to the right, and vice versa). Thus cells that normally are not neighbors come to lie next to each other, and when the cells from these normally non-adjacent tissues interact during blastema formation, the differences in their positional information codes are recognized and supernumerary structures are induced to form. Although strange looking structures can form, their growth and appearance are predicted by the Polar Coordinate Model in that the extra structures are what normally would occur between the graft and host cells [13].

Intercalation of new pattern also can be demonstrated by reprogramming of their positional information by treatment with retinoic acid, such that anterior cells acquire posterior positional information [14]. When the cells within posterior wounds are treated with retinoic acid, no intercalation occurs and no supernumerary pattern is induced because the cells already have posterior information. In contrast, cells within anterior wounds are reprogrammed by retinoic acid to become posterior cells that then interact with the surrounding anterior cells and multiple supernumerary limb structures are induced to form [13] (see also [15]). Taken together, these experiments predict that there is a 2D grid of positional information that underlies all epithelia and wraps around the internal tissues of the limb; i.e. the loose connective tissues of the dermis and the internal tissues of the limb (see [10]).

The importance of positional information for regeneration has been demonstrated directly with the Accessory Limb Model [16,17]. This model was developed as a gain-of-function model for regeneration (Figure 1) based on early experiments demonstrating that ectopic

limbs could be induced to form from wounds on the side of the arm of salamander [18,19]. When a full thickness skin wound is made surgically, the wound heals by forming a wound epithelium, and eventually the skin is regenerated without scar formation. It has been known for decades that regeneration requires nerve signaling above a threshold level [10,20]. The threshold level of innervation is achieved by surgically deviating the brachial nerve to the skin wound where it interacts with the wound epithelium to recruit cells via migration from the surrounding connective tissues [16,21]. This leads to the formation of an ectopic blastema that is equivalent in terms of cellular behaviors and gene expression to a blastema that forms in response to limb amputation [17].

Although the mechanistic details of the neuro-epithelial interactions leading to the formation of a regeneration-permissive apical epithelium are unknown, it appears that they involve growth factor signalling from the injured nerves [22] that induced epigenetic modifications of the wound epithelium via de novo DNA methylation [23]. The recent discovery of the involvement of FGF (fibroblast growth factor) and BMP (bone morphogenetic protein) signaling [24–26] are encouraging in terms of being able to tease apart the specifics of how this early step in regeneration is controlled. Although an ectopic blastema can be induced to form in response to signaling from the deviated nerve and wound epithelium, it lacks the positional information to make a new limb since the cells recruited from around the wound site all share similar positional codes. The Polar Coordinate Model predicts that formation of an ectopic limb requires cells with positional information from opposite sides of the limb, and this is accomplished in the Accessary Limb Model by grafting a piece of skin from the posterior side of the arm to a wound on the anterior side (or vice versa). With the addition of cells with different positional information, the induced blastema now is able to regenerate an entire new limb (Figures 1, 2). Thus the Accessory Limb Model demonstrates that regeneration occurs via a series of steps that are initiated by injury. At each step, additional signals are required in order to progress to the next step. Failure to progress at any step thus will result in the failure to regenerate. In terms of the failure of humans to regenerate an amputated limb, it is possible that many of the steps can occur; however, at least one of the required steps must fail. Thus the challenge for inducing human regeneration is to identify the required steps and then test each for success or failure, starting at the beginning and proceeding until the final required step has occurred.

The Accessory Limb Model as an assay for signals that control the regeneration blueprint

In addition to demonstrating the step-wise progression of regeneration, the Accessory Limb Model can be used to assay for the signals that allow for progression from one step to the next. This approach has been successful in identifying signals that can substitute for the early steps associated with signaling from the deviated nerve. Putative signaling factors can be delivered to the skin wound by implanting gelatin beads soaked in these factors, and then assaying for induction of an ectopic blastema in the absence of a deviated nerve. This approach has led to the identification of several cocktails of human growth factors (mainly FGF and BMP) that can induce an ectopic blastema that can form an ectopic limb when a piece of skin from the opposite side of the limb is grafted along with the growth factor bead

[24–26]. In these experiments, gelatin beads that are between 300–600 μm in diameter [27] were soaked in various mixtures of signaling molecules, and then were grafted beneath the newly formed wound epithelium. The factors then diffused out of the beads and interacted with the basal keratinocytes of wound epithelium and the cells of the mesenchymal tissues at the wound site. Since many of these growth factors that can induce ectopic blastema formation also are produced by injured axolotl nerves [22,24], it is presumed that the factors are release from the nerves and function the same as when they are released from the implanted gelatin beads. Regardless of whether these are the endogenous nerve-associated factors that function when an axolotl arm is amputated, they can activate the appropriate signaling cascade(s) to achieve the same outcome of growing an entire new arm. The observation that human growth factors can induce axolotl blastema formation is evidence that the early signaling pathways for regeneration are conserved between salamanders and humans, and that regenerative failure in humans is not a consequence of not being able to provide blastema-inducing signals.

With regards to the later steps of regeneration that are dependent on positional information, the Accessory Limb Model also has been used to assay for signals associated with the posterior skin graft that can induce formation of limb structures [6]. When a piece of skin is grafted from the opposite side of the limb, the graft contains both cells and extracellular matrix (ECM). As discussed above, the cells with positional information are localized in the loose connective tissues, and since these cells also synthesize the ECM we hypothesized that the information would be associated with the ECM. We tested this by treating posterior skin grafts with urea to remove the cells prior to grafting to anterior skin wounds in the Accessory Limb Model. As anticipated, cells in ectopic blastemas responded to signals associated with the cell-free ECM and formed ectopic limb structures (Figure 3A). The ability of cell-free axolotl limb ECM to control pattern formation was position-specific in that posterior ECM induced pattern formation in anterior blastemas; whereas, anterior ECM did not, but rather it inhibited blastema formation entirely [6].

Given the role of sulfated glycosaminoglycans, heparan sulfate proteoglycans (HSPGs) in particular, in the regulation of growth factor/morphogen signaling, we tested whether the ECM pattern-forming activity detected by the Accessory Limb Model assay was dependent on HSPG by treating the ECM with heparan lyase prior to grafting. The ability to induce ectopic pattern was reduced but not eliminated by removal of HSPG; however, the anti-regeneration activity of anterior ECM grafted into anterior wounds was dependent on HSPG. Given the high affinity of HSPG for growth factors, it therefore is possible that the bioactivity was a consequence of the ECM being either a source or sink, or both, for growth factors/morphogens. There is evidence for both of these functions. The activity associated with anterior ECM that inhibits blastema formation appeared to be a consequence of the presence of a factor that is bound to the ECM in an HSPG-dependent manner since treatment with heparan lyase prior to grafting removed this activity. Similarly, treatment with high concentrations of salt removed this activity, which is consistent with the hypothesis that there is a factor that is bound to the ECM that can be removed. Conversely, the hypothesis that HSPG-rich ECM functions as a sink that interferes with endogenous growth factor signaling pathways is consistent with the observation that a synthetic collagen-HSPG hydrogel induced pattern formation in ectopic blastemas (Figure 3C). These implanted

hydrogels are viscous and amorphous, and therefore do not induce pattern formation by providing topographical cues to the axolotl blastema cells. Presumably the hydrogel sequesters growth factors that the cells use to communicate with each other, and the induced pattern arises as a consequence of changes in the normal distribution of growth factor signaling that is mediated by specific HSPGs in the positional information grid.

Regardless of whether HSPGs function to deliver growth factors or to sequester endogenous growth factors, it is well established that there is a specificity to growth factor binding that is associated with the patterns of HSPG sulfation [3,4]. If the spatially distinct signaling activities of the ECM are meditated by spatially unique HSPG sulfation patterns, then presumably the expression of the enzymes responsible for generating the sulfation patterns must be spatially and/or temporally regulated. This turns out to be the case for three of the six sulfotransferases that are expressed in axolotl limb skin cells [6]. This differential expression of sulfotransferases is predicted to result in qualitative and quantitative differences of sulfation across the anterior/posterior limb axis that in turn are predicted to differentially regulate growth factor signaling (specifically FGF signaling) between the anterior and posterior region of the blastema [3,4,6].

Mammalian signaling molecules can induce a regeneration response in the Accessory Limb Model

Mammals have limited regenerative ability, and in most cases, injuries (e.g. limb amputations) do not regenerate, but rather form scar tissue. This non-regenerative response to injury implies that at least one of the necessary steps for regeneration does not occur, or is not functional in mammals. The essential role of positional information in salamander limb regeneration raises the possibility that a lack of information, or an inability to access this information, could account for regenerative failure in mammals. To test whether or not there is positional information in mammalian tissues, we grafted ECM from mouse limb skin into the axolotl Accessory Limb Model. Positional information cannot be assayed directly in the mouse because it does not regenerate; whereas, this can be done with in the axolotl since if the appropriate signals are provided, the cells can respond and regenerate limb structures. Is possible to assay for this activity associated with the mouse ECM since the signaling activity is not dependent on the survival of mammalian cells in an axolotl host.

Experiments using the Accessory Limb Model as an assay for positional information in mouse limb skin ECM have yielded much the same results as observe for axolotl limb skin ECM [6]. Grafts of posterior (but not anterior) limb skin ECM from neonatal mice induced pattern formation in anterior ectopic axolotl blastemas. This signaling was transient, and was detected only between postnatal days three to seven. Grafts of ECM from limb skin from E11.5 mouse embryos had no effect on blastema formation; whereas, postnatal day 1 grafts inhibited blastema formation. After the developmental window during which pattern formation was induced (PN3-7), ECM grafts did not effect blastema formation or induce pattern formation. The ability of PN3/4 ECM grafts to induce pattern formation was dependent on HSPG in as much as heparan lyase pretreatment eliminated the ability to induce pattern formation. These date are consistent with the hypothesis that the underlying

mechanisms of embryonic and regenerative development are conserved, and used by both salamanders and humans.

Engineering the positional information blueprint

Ultimately, regeneration occurs as a result of the orchestrated regulation of conserved signaling pathways. It now has become appreciated that the ability of these critical pathways (e.g. FGF) to be activated is dependent on specific sulfation modifications of HSPGs in the ECM. The importance of these modifications has been demonstrated for both developing and regenerating limbs [6,9]. These modifications are stably encoded in the ECM such that decellularized ECM can participate in regulating the response of cells to these growth factors [3,4,6]. Therefore, the specific chemical modifications of the ECM are equally important as the presence or absence of the ligands needed to activate the receptor and downstream intracellular signaling cascades. One strategy to induce a regenerative response would be to provide the necessary ligands to the wound at the right time and concentrations without triggering off-target effects. An alternative approach would be to engineering a “smart” ECM with the appropriate sulfation codes for these ligands. This engineered matrix would provide the appropriate spatial arrangements of codes that in turn would bind endogenous ligands and control their spatial and temporal activities. The feasibility of this approach is evident from studies of FGF signaling in which the sulfation affinity codes for different FGF ligands already have been discovered [3,4]. This approach would be widely applicable since sulfated glycosaminoglycans are implicated in the regulation of signaling by a number of growth factors and bioactive molecules in addition to FGFs (see [4]). This approach builds on the convergence of engineering and developmental biology to enable the engineering of an ECM that is tuned for the regulation of pro-regenerative growth factor signaling.

This strategy for inducing human regeneration is encouraging given the many levels of conservation of developmental mechanisms between axolotls and mammals. For example, human growth factors can induce blastema formation in axolotls [24–26]. Although there may be variations in protein sequences between these distant species, the underlying ligand-receptor interactions, as well as downstream signaling cascades must be functionally conserved. In addition, HSPG-mediated positional signaling appears to be conserved between axolotl and mammals since pattern formation in axolotl blastemas can be induced by ECM molecules from mammals (decellularized mouse dermis and purified porcine HSPG; Figure 3C). Finally the Accessory Limb Model demonstrates that although the steps leading to regeneration can occur (e.g. when you amputate an axolotl limb it regenerates), they will only occur if the appropriate signals are provided (e.g. nerve factors/FGF-BMP cocktail). Among the signals are those associated with the ECM that provide the positional information necessary for the blastema cells to reform the patterned structures of the limb. The Accessory Limb Model thus demonstrates that although a regenerative response may not occur, it does not mean that it cannot occur if the appropriate signals are provided. The lesson learned as we move forward towards the goal of induced human regeneration is that the lack of a regenerative response is not the same as a lack of the ability to regenerate.

Future works

The future for regenerative engineering is bright. The technologies and techniques for engineering the positional information grid are already available. Advances in glycobiology will provide a map of sulfation patterns that will enable us to control growth factor signaling to initiate and navigate the regeneration cascade leading to human regeneration.

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Lay Summary

Just as the building of a house requires a blueprint, the rebuilding of lost or damaged body parts through regeneration requires a set of instructions for the assembly of the various tissues into the right places. Recent studies provide evidence that this blueprint is encoded in part by an information-rich extracellular matrix (ECM) within the loose connective tissues that controls the behavior of regeneration-competent cells. The ability of the ECM to do this is associated with sugar-rich macromolecules that have specific spatial and temporal modifications of sulfation patterns. This mechanism for controlling pattern formation appears to be conserved between salamanders and mammals, and thus the next challenge for inducing human regeneration is to identify and understand the biology of the connective tissue cells and the ECM that they synthesize.

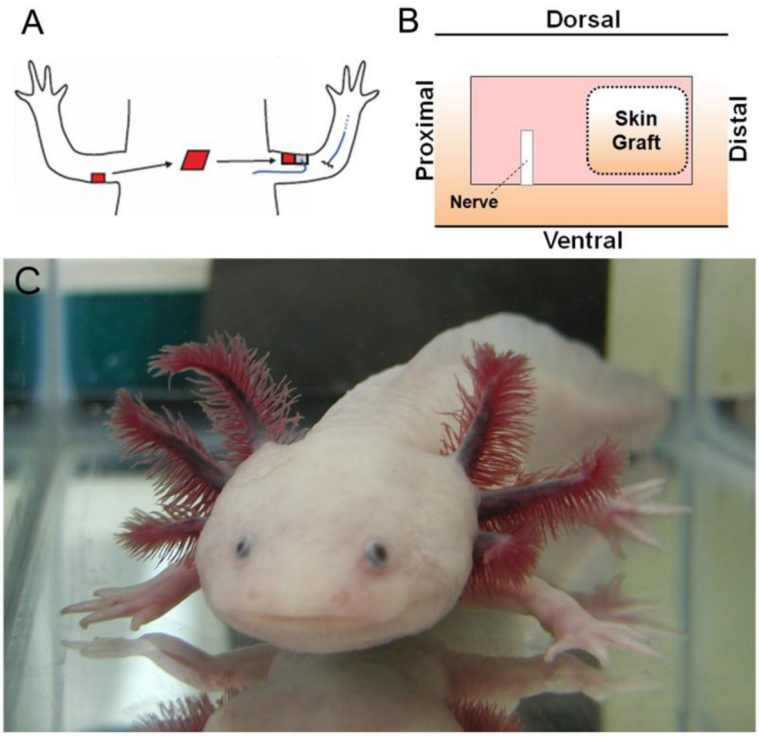

Figure 1. The Accessory Limb Model as a gain-of-function assay for the signals controlling regeneration

Making an accessory axolotl limb [16]. (A) Ectopic limbs with normal pattern can be induced to form in response to a wound to which a nerve (blue) is surgically deviated, and a piece of skin (red) from the opposite side of the limb (posterior) is grafted into the host site (anterior) as indicated by the black arrows. (B) Cartoon illustrating the final arrangement of wound, deviated nerve, and skin graft that will result in formation of an ectopic limb. Signaling associated with the wound, in addition to signals from the deviated nerve and the skin graft are sufficient to generate a supernumerary limb (two left arms) as shown in (C). Modified with permission from Bryant and Gardiner [5].

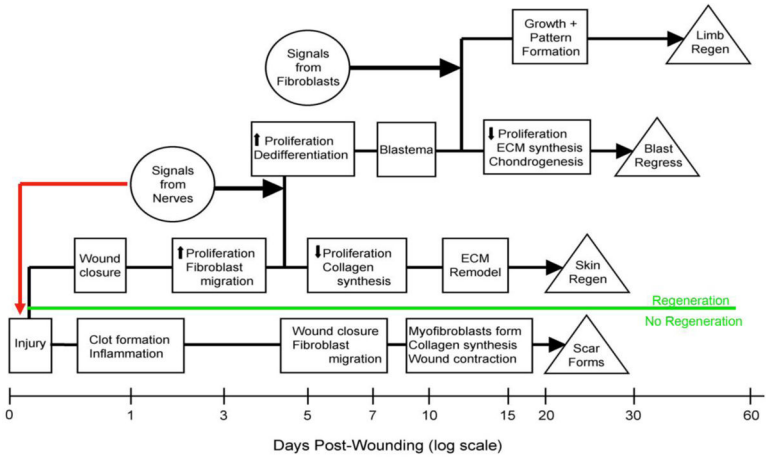

Figure 2. Regeneration is a step-wise process

The sequence of steps that occur in the accessory limb model [16] illustrated in Figure 1 also can be illustrated as a flow diagram. In response to injury, the wound either regenerates (above the green line) as occurs in the axolotl, or it forms a scar (below the green line) as occurs in humans. Many of the same steps occur in both regenerating and non-regenerating wounds (e.g. inflammation and fibroblast migration). The different outcomes are a consequence of proregenerative signals associated with injury in the Accessory Limb Model. Signals from the wound function early (red arrow) and at later times (black arrow) to recruit connective tissue cells that dedifferentiate and proliferate to form the blastema. If no further signals are provided, the induced blastema eventually stops growing and is integrated into the host tissues. If additional signals are provided by fibroblasts in a skin graft (or ECM graft) from the side of the limb opposite to the wound site, the ectopic blastema continues to grow and forms an ectopic limb.

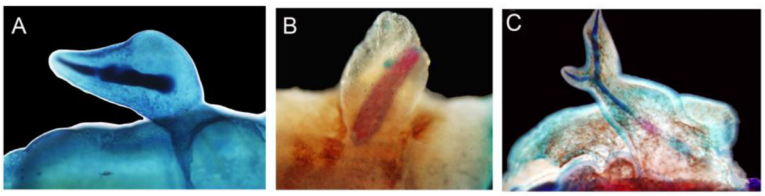

Figure 3. Ectopic limb structures can be induced by cell-free ECM and purified ECM components from both salamanders and mammals

Ectopic blastemas that are induced to form in the Accessory Limb Model [16] will not form differentiated limb structures unless they also are provided positional information, e.g. by grafting skin from the side of the limb that is opposite the site of the wound. Ectopic limb structures also can be induced by grafting (A) decellularized ECM from axolotl limb skin, (B) decellularized ECM from neonatal mouse limb skin, or (C) an artificial ECM composed of porcine heparan sulfate proteoglycan (HSPG) and bovine collagen. Whole mount preparations are stain with alcian blue (cartilage) and alizarin red (bone).