J Endocrinol Reprod 12 (2008) 2: 67 - 72

Assessment of extracellular matrix remodeling during tail regeneration in the lizard *Hemidactylus flaviviridis*

V.V. Nambiar, I.Y. Bhatt, P.A. Deshmukh, R.R. Jape, P.N. Jivani, H.R. Kavale, S.S. Prakashkar and A.V. Ramachandran

Division of Developmental and Reproductive Endocrinology, Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara - 390 002, India

SUMMARY

Epimorphic regeneration, as exemplified by lizard tail, involves the formation of regeneration blastema as a pre-requisite for replacement growth. The formation of blastema is preceded by many regressive changes like tissue demolition, histolysis, inflammation, wound closure, dedifferentiation, cell migration and proliferation. The ECM components of a normal tail help maintain the differentiated state of the constituent heterogeneous tissue and tend to prevent cell migration and proliferation. Since the formation of a regeneration blastema involves many key cellular events like dedifferentiation, migration of dedifferentiated and other nomadic cells and cell proliferation, extensive reorganization of the extracellular matrix is likely to be a major event during post-autotomy period. To this end, total GAG content and gelatinase (MMP-2 & 9) activity were assayed during the first week post-caudal autotomy. The effect of MMP inhibition by doxycycline was also evaluated in terms of the number of days taken for wound closure and blastema formation as well as the length of detail regenerated at the end of twenty days from the time of initiation of growth. Significant progressive decrement in total GAG content up till 96 hours followed by a significant increment at 120 and 144 hours, together with a biphasic increment in gelatinase activity (first during 24-48 hours and second during 96-144 hours) have been recorded. The increased gelatinase activity and the decreased GAG content in the first 96 hours are suggestive of breakdown of proteoglycans and collagens (type IV, V, VII & X). The second phase of increase in GAG content is related with synthesis of hyaluronate, favoring dedifferentiation, proliferation and migration of cells. MMP inhibition by doxycycline significantly retarded tail regeneration. From these results it is concluded that ECM remodeling is crucial in the immediate post-autotomy period for the formation of an effective blastema and that MMPs play a crucial role in the same.

Key words: ECM remodeling, GAGs, lizard, MMPs, regeneration

Introduction

Regeneration can be defined as the ability to reproduce relatively complicated organs or structures after they have been lost through trauma or other causes. Regeneration in lower invertebrates is essentially a mode of asexual reproduction while in higher invertebrates and vertebrates it is an adaptive mechanism essentially for replacement of lost part with functional exigency. Regenerative medicine has a challenging task of unraveling the secrets of how body parts can regenerate. The ability to shed or readily lose appendages as a method of escape from enemies, as in the case of lizard tail, is known as autotomy (self-amputation). The regeneration seen in higher vertebrates is epimorphic and the tropical lizard *Hemidactylus flavivirdis* is an excellent example showing epimorphic regeneration of its tail. Epimorphic regeneration is characterized by post-autotomy regressive (wound healing and dedifferentiation) and progressive (blastema and differentiation) phases. It is characterized by non-scarring type of wound healing and the formation of a regeneration blastema (akin to the embryonic tail bud) and redifferentiation and growth of tail (Ramachandran, 1996).

Though the formation of a blastema is essential for replacement growth to occur, the immediate post-autotomy period is crucial for its establishment. Thus, the present study addresses an aspect of molecular intricacy in the immediate post-autotomy phase, which could aid in setting up an appropriate environment for the initiation of regeneration. A number of synchronized inter-related molecular events involving cytokines, growth factors, hormones and modifications of extracellular matrix (ECM) are likely to trigger the adaptive changes crucial for the early regressive phase of regeneration. It is known that there is loss of tissue organization at the cut end of the

Correspondence to be addressed to: Dr. A.V. Ramachandran, Ph.D., Email: *avrcn@rediffmail.com

organ leading to liberation of cells and their dedifferentiation (see Stocum, 1995, 2006). It is likely that remodeling of extracellular matrix (ECM) can help liberated cells re-enter the cell cycle and lose their phenotypic specializations, and change their pattern of gene activity to facilitate regeneration.

The ECM is degraded by acid hydrolases and matrix metalloproteinases (MMPs) (see Stocum, 2006; Carlson, 2007). Macrophages, neural cells, osteoclasts and epidermal cells can be the sources of MMPs (see Clark, 1996; Stocum, 2006). MMPs degrade a variety of ECM substances and function as ECM clearing enzymes favoring cell migration, invasion, proliferation and morphogenesis (Vu and Werb, 2000).

Glycosaminoglycans (GAGs), anionic polysaccharides, principally constituents of ECM but also present on the surface of some cells, are reported to assist proliferation in general and tumor formation specifically because of their capacity to bind and modulate a variety of proteins including growth factors, cytokines and proteases (Smetsers *et al.*, 2003). There is specific GAG composition at different levels of anlagen and at different embryonic stages, suggesting heterogeneous composition of ECM during development (Becchetti *et al*, 1988; Bulow and Hobert, 2006).

In this context, the present study was aimed at assessing the role of ECM remodeling as a pre-requisite for dedifferentiation and cell migration. ECM remodeling has been investigated adopting actual measurement of MMP activity and the effect of MMP inhibitor Doxycyclin on the progression of regeneration as well as measurement of total GAG material every 24 hours during the first six days post-autotomy.

Materials and Methods

Lizards

Adult lizards, *Hemidactylus flaviviridis*, procured from a local animal dealer, were maintained in wooden cages measuring 18" x 15" x 10" with transparent sliding glass on one side and ventilated on three sides with windows covered by wire net. Lizards weighing 10 – 12 g and snout – vent length of 8 – 10 cm were selected and housed in the wooden cages, 8 animals per cage, and fed on cockroach nymphs *ad libitum* with provision for water *ad libitum*. The experiments were carried out in the summer months with average cage temperature of 33 - 34° C.

Experimental set- ups

SET – UP 1: Estimation of total GAGs and MMP

Schedule 1: Estimation of total GAGs

32 lizards were taken and their tails were autotomized at 8:00 hr. The autotomized end of the tail stump of four lizards each was taken at zero, 24, 48, 72, 96, 120 and 144 hr post- autotomy for the estimation of total GAG content adopting the method of Gold (1979).

Schedule 2: Estimation of matrix metalloproteinase (MMP)

32 lizards were autotomized at 8:00 hr. The cut end of the tail stump of four lizards each was taken at zero, 24, 48, 72, 96, 120, 144 and 168 hr post- autotomy and MMP, specifically gelatinase activity, was estimated by the modified method of Anson (Anson, 1938; Crewther, 1952). The enzyme activity was expressed as µg tyrosine released per hour.

SET – UP 2: MMP inhibition

Doxycyclin tablets, Microdox DT, manufactured by Microlabs Ltd., Unit III, were used for MMP inhibition. Doxycyclin was dissolved in amphibian saline.

8 lizards were selected and their tails autotomized at 8:00 hr. Four of these lizards were administered with 0.1 ml Doxycylin (50 µg/g) intraperitoneally daily for nine days from the day of autotomy and served as experimentals, while the remaining four lizards were administered with vehicle alone and served as controls.

The effect of MMP inhibition was assessed in terms of time taken to attain the various arbitrary stages of regeneration – wound healing, blastema and initiation of growth- as well as the length of tail regenerated at the end of 20 days.

Results

Total GAG content

Total GAG content showed continuous decrease from 24 hr post-autotomy to reach minimal level by 96 hr. The GAG content then increased significantly to supra-normal levels at 120 hr and 144 hr (Fig. 1).

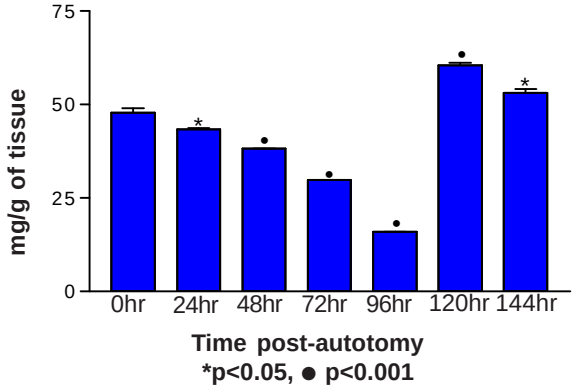

Fig.1. Changes in total GAG content post - caudal autotomy

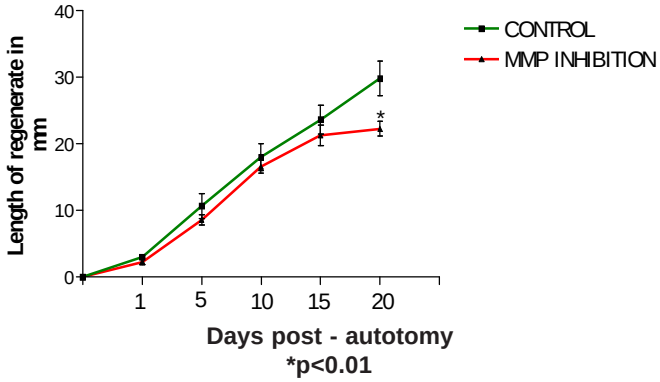

Fig.3 Length of tail replaced in control and Doxycylin treated lizards

Gelatinase activity (MMP-2 & MMP-9)

MMP-2 and MMP-9, measured as gelatinase activity, showed significant increase within 24 hr of autotomy and the enzyme activity remained high till 144 hr to decrease to near normal levels at 168 hr (Fig. 2).

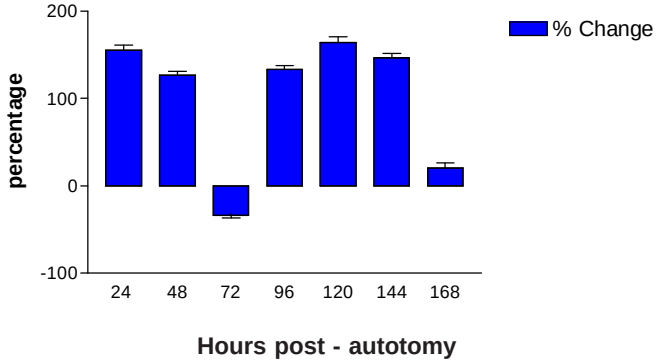

Fig. 2. Percentage increase or decress in gelatinase activitiy post-caudal autotomy

MMP inhibition

Inhibition of MMP activity by Doxycyclin showed significant delay in the number of days taken to complete wound healing from a blastema and initiate growth (Table 1). The delay in the initiation of growth was reflected in the reduced regenerative replacement seen at the end of 30 days post-autotomy in Doxycyclin-treated lizards (Fig. 3).

Table 1: Number of days taken to attain various stages of regeneration in control and Doxycyline treated lizards. Values are Mean ±SD.

Set	WH	BL	IG
Control	7.0 ± 0.012	9.0 ± 0.57	10.66 ± 0.33
Doxycycline	10.33 ± 0.33 *	12.0 ± 0.48*	13.0 ± 0.39*

** P < 0.05*

The maximum delay in the growth rate in MMP inhibitor treated animals was seen during the 15 – 20 day period as compared to a constant growth rate in the control animals (Fig. 4).

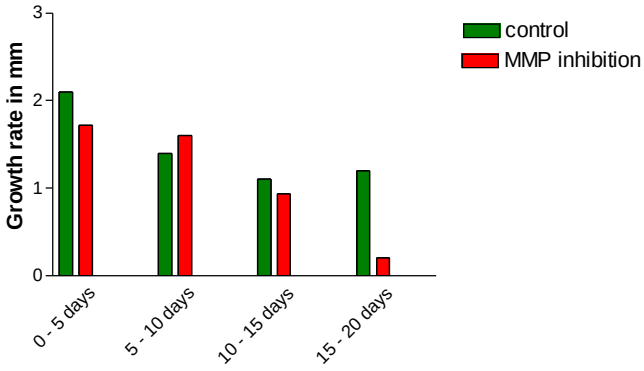

Fig. 4. Growth rate of tail in control and Doxycyclin treated lizards in blocks of 5 days

Discussion

Crucially timed breakdown of extracellular matrix (ECM) is essential for embryonic development, morphogenesis, reproduction, and tissue resorption and remodeling. ECM is not a mere cell anchorage scaffold but houses many covert latent biological functions as in embryonic development, tissue remodeling, wound healing and repair as well as in pathophysiology. Two main classes of extracellular macromolecules which make up the matrix are (i) polysaccharide chains of the class called glycosaminoglycans (GAGs), which are usually found covalently linked to protein in the form of proteoglycans, and (ii) fibrous proteins, including collagen, elastin, fibronectin, and laminin, which have both structural and adhesive functions. The regressive phase of epimorphic regeneration, characterized by cell dedifferentiation, cell migration and cell proliferation, is likely to involve

remodeling of extracellular matrix, to facilitate regeneration cascade. Hence, the present study was taken up to assess the alterations in the extracellular matrix in the initial phase of regeneration.

Biphasic increase in gelatinase activity, coupled with significant decrease in total GAG material, is the hallmark observations of the present study. The initial two-fold increase in gelatinase activity was in the immediate 24 – 48 hr post-autotomy, with sharp decline at 72 hr, followed by elevated level persisting between 96 – 144 hr. The initial up-regulation of gelatinase activity could be related with epithelial cell migration for wound closure and prevention of laying down a basement membrane favoring free epithelial-stump tissue interactions essential for regenerative induction. The second phase of increased enzyme activity could be correlated with ECM remodeling supporting dedifferentiation, migration and proliferation of cells and generation of a mesenchyme-rich blastema. Though timed-up regulation of gelatinase activity has been seen in regenerating *Xenopus* limb (Chernoff *et al*., 2000) as well as during the course of regeneration of airway epithelium (Mohan *et al*., 2002), liver (Ole *et al*., 2006; Verbesey *et al.*, 2006) and lung (Xu *et al*., 1999; Puchelle *et al*., 2001), a biphasic increase in MMP activity as seen in the present study is also seen during Axolotl limb regeneration (Yang et *al*., 1999; Santosh *et al.*, 2008). It has been generally accepted that ECM molecules rapidly turn over during processes involving tissue remodeling, such as wound healing, metamorphosis and regeneration, and specifically MMP2 (gelatinase-a) and MMP9 (gelatinase-b) seem to be the principal players (see Stocum, 2006; Atala *et al.*, 2008). The MMPs play a major role in the degradation of the ECM and, consequently, the degradation of stump ECM destabilizes the differentiated state and acts as a trigger for dedifferentiation by liberating cells. The dedifferentiated cells then proliferate and form a blastema. MMP2 (gelatinase-a) and MMP9 (gelatinase-b) hydrolyze gelatin, proteoglycan and collagens (types IV, V, VII, and X). Other hydrolytic enzymes like acid phosphatase and β-glucuronidase have also been shown to be upregulated in the post-autotomy periods in *H. flaviviridis* (Shah and Chakko, 1966; Shah and Hiradhar, 1976). There is an experimental limitation in isolating the role of MMP2 and MMP9 since the current experimental protocol is based on total gelatinase assay using gelatin as substrate. Despite this methodological limitation, the results can be discussed in the light MMP2 and MMP9 owing to substrate (gelatin) specificity. It has been reported that MMP-9 and, to a lesser extent, MMP-2 are products associated with macrophage invasion (Longo *et al*., 2002) and wound epidermis, macrophages, osteoclasts and neuritic tissue are the possible sources of these enzymes during regeneration (Stocum, 2006; Atala *et al.*, 2008). This might, at least in part, account for the observed increase in gelatinase activity at 24-48 hr post-autotomy, initiating degradation of the ECM.

There was significant decrease in total GAG content till 96 hr post-autotomy followed by steady increase after 96 hr. This suggests breakdown of collagen, laminin, dermatan sulphate, etc., and release of ECM-bound growth factors. Concomitant increase in gelatinase activity reinforces this inference. Interestingly, there was an increase in total GAG material after 96 hr and this can be attributed to upregulation of hyaluronate, tenacin and fibronectin, generating an ECM conducive for cell migration and proliferation. Corroborative evidence in favor of this contention can be had from the recorded nature of extracellular material during fetal wound healing. Fetal wound repair, devoid of scarring, is very much similar to epimorphic regeneration. The level of hyaluronate has been found to be significantly elevated in the fetal wound environment with steady decline during the adult life. Similarly, the hyaluronate receptor was also found to be upregulated in fetal condition, and hyaluronate is the most frequent glycosaminoglycan (GAG) found in the ECM of the mesenchymal tissues during the initial stages of embryonic as well as regenerative growth and development. Hence, its presence has been associated with proliferative processes, and its decrease with cell differentiation (see Garg and Longekar, 2000; Stocum, 2006; Atala *et al*., 2008). *In vitro* studies have shown that hyaluronate alters the profile of inflammatory mediators such that the balance between cell matrix synthesis and degradation is shifted away from degradation (Smith *et al*., 1987; Frean *et al*., 1999; Takahashi *et al*., 1999; Nonaka *et al*., 2000; Moreland, 2003). On the basis of this background information on the beneficial role of hyaluronate, and on the basis of the results from the present study, it can be speculated that the increase in hyaluronate after 96 hr favors ependymal and neuronal outgrowth and, subsequently, dedifferentiation, cell migration and proliferation, all essential for the formation of a robust blastema.

Remodeling of the ECM is crucial for dedifferentiation and blastema formation. This remodeling is achieved through the role of MMP's, lysosomal acid hydrolases, cathepsins, beta glucoronidase and acid phosphatase (Shah and Chakko, 1966; Shah and Hiradhar, 1976; Stocum, 2006; Atala *et al*., 2008). The role of MMPs in remodeling the matrix in regenerating system has been

reported in other models of epimorphic regeneration (see Stocum, 2006; Carlson, 2007; Atala *et a*l., 2008). To this effect, doxycycline, a known inhibitor of MMP, was employed to study the time course of attainment of various stages of regeneration and to assess the per day growth rate following its administration. Inhibition of MMP activity by doxycycline showed significant delay in the attainment of wound healing, blastema formation and initiation of growth. The relevance of matrix metalloproteinase (MMP) inhibition by doxycycline, an effective MMP inhibitor, was tested in a rat model of extensive myocardial infarction (MI) and left ventricular (LV) dysfunction. Doxycycline was shown to inhibit the growth of engrafted melanoma and result in reduced expression of MMP-2, MMP-9, and vasculogenesis (Sun *et al*., 2007), and prevent upregulation of MMP2 and MMP9 in ischemia reperfusion injury (Roach *et al*., 2002). In other experiments on rats doxycycline prevented collagen degradation by inhibition of MMPs in a model of subcutaneous injury, and decreased MMP activity and failed to attenuate scar thinning in models of myocardial infarction and left ventricular dysfunction (Lamparter, 2002; Tessone *et al*., 2005). Thus, the presently observed delay in the initiation of regeneration and retarded growth rate highlight the importance of MMPs and ECM remodeling by way of collagen degradation and hyaluronate synthesis in the crucial regressive phase of epimorhic regeneration.

References

Anson ML (1939) The estimation of pepsin, trypsin, papain and cathepsin with haemoglobin. *J Gen Physiol* 22: 78-89.

Atala A, Laza R, Thompson J, Nerem R (2008) *Principles of Regenerative Medicine*. Academic Press, p 1448.

Becchetti E, Evangelisti R, Stabellini G, Pagliarini A, del Borrello E, Calastrini C, Carinci P (1988) Developmental heterogeneity of mesenchymal Glycosaminoglycans (GAG) distribution in chick embryo lung anlagen. *Am J Anat* 181: 33-42.

Bulow HE, Hobert O (2006) The molecular diversity of glycosaminoglycans shapes animal development. *Ann Revf Cell DevBiol* 22: 375-407.

Carlson B (2007) *Principles of Regenerative Biology*. Academic Press, New York, p 400.

Chernoff EAG, O'hara CM, Bauerle D, Bowling M (2000) Matrix metalloproteinase production in regenerating axolotl spinal cord. *Wound Repair and Regeneration* 8: 282-291.

Clark RAF (1996) *The Molecular and Cellular Biology of Wound Repair*. Plenum Press, New York, p. 611.

Crewther WG (1952) The precipitation of gelatin by ethanol and its use in estimation of proteolytic activity. *Aust J Sci Res, Ser B* 5: 290-301.

Frean SP, Abraham LA, Lees P (1999) *In vitro* stimulation of equine artilcular cartilage proteoglycan synthesis by hyaluronan and carprofen. *Res Vet Sci* 67: 183- 190.

Garg HG, Longekar MT (2000) *Scarless Wound Healing*. Informa Health Care, p 335.

Gold EW (1979) A simple spectrophotometric method for estimating glycosaminoglycan concentrations. *Anal Biochem* 99: 183-188.

Lamparter S (2002) Doxycycline and tissue repair in rats. *J Lab Clin Med* 139: 295-302.

Longo GM, Xiong W, Greiner TC, Zhao Y, Fiotti N, Baxter BT (2002) Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *J Clin Invest* 110: 625-632.

Mohan R, Chintala SK, Jung JC, Villar WVL, *et al.* (2002) Matrix metalloproteinase gelatinase B (MMP 9) coordinates and affects epithelial regeneration. *J Biol Chem* 277: 2065- 2072.

Moreland LW (2003) Intra-articular hyaluronan (hyaluronic acid) and hyalans for treatment of osteoarthritis: Mechanisms of action. *Arthritis Res Ther* 5:54-67.

Nonaka T, Kikuchi H, Ikeda T, Okamoto Y, Hamanishi C, Tanaka S (2000) Hyaluronic acid inhibits the expression of u-PA, PAI-1 and u-PAR in human synovial fibroblast of osteoarthritis and rheumatoid arthritis. *J Rheumatol* 27: 997-1004.

Olle EW, Ren X, McClintock SD, Warner RL, Deogracias MP, Johnson KJ, Colletti LM (2006) Matrix metalloproteinase-9 is an important factor in hepatic regeneration after partial herpatectomy in mice. *Hepatology* 44: 540-549.

Puchelle E, Vargaftig BB (2001) Chronic obstructive pulmonary disease: and old disease with novel

concepts and drug strategies. *Trends Pharmacol Sci* 22: 495-497.

Ramachandran AV (1996). Biochemistry and metabolism of lizard tail regeneration. *J Anim Morphol Physiol* 43: 1-13.

Roach DM, Fitridge RA, Laws PE, Millard SH, Varelias A, Cowled PA (2002) Up-regulation of MMP-2 and MMP-9 leads to degradation of type IV collagen during skeletal muscle reperfusion injury; Protection by the MMP inhibitor, doxycycline. *Eur J Vasc Endovasc Surg* 23: 260-269.

Santosh N, Al-Shibani N, Labban N, Rao N, Li B, Windsor LJ, Song F, Stocum D (2008). Expression of matrix metalloproteinase (MMPs) during axolotl limb regeneration. *Dev Biol* 319: 559.

Shah RV, Chakko TV (1966) Histochemical localization of acid phosphatase in the adult normal and regenerating tail of house lizard, *Hemidactylus flaviviridis*. *J Anim Morphol Physiol* 13: 169-188.

Shah RV, Hiradar PK (1976) Possible role of beta-glucoronidase on the normal and regenerating tail of the house lizard, *Hemidactylus flaviviridis*. *ActaAnat (Basel)* 95: 508-517.

Smetsers TFCM, van de Westerlo EMA, tan Dam GB, Clavigs R, Versteeg EMA, van Geloof WL, Veerhkamp JH, van Munijen GNP, van Kuppeevelt TH (2003) Localization and characterization of melanin associated glycosaminoglycans: Differential expression of chondroitin and heparan sulfate epitopes in melanoma. *Cancer Res* 63: 2965-2970.

Smith MM, Ghosh P (1987) The synthesis of hyaluronic acid by human synovial fibroblasts is influenced by the nature of the hyaluronate in the extracellular environment. *Rheumatol Int* 7: 113-122.

Stocum DL (2006) *Regenerative Biology and Medicine*. Academic Press, New York, p. 448.

Sun B, Zhang S, Zhang D, Yin X, Wang S, Gu Y and Wang Y (2007) Doxycycline influences microcirculation patterns in B16 melanoma. *Exp Biol Med (Maywood)* 232: 1300-1307.

Takahashi K, Goomer RS, Harwood F, Kubo T, Hirasawa Y, Amiel D. (1999) The effects of hyaluronan on matrix metalloproteinase-3 (MMP-3), interleukin-1 beta(IL-1 beta), and tissue inhibitor of metalloproteinase-1 (TIMP-1) gene expression during the development of osteoarthritis. *Osteoarthritis Cartilage* 7:182-190.

Tessone A, Feinberg MS, Barbash IM, Reich R, Holbova R, Richman M, Mardor Y, Leo J (2005) Effect of matrix metalloproteinase inhibition by doxycycline on myocardialhealing and remodeling after myocardial infarction. *Cardiovas Drugs Ther* 19: 383-390.

Verbesey JE, Simpson MA, Zurakowski D, Dwyer W, Puder M, Pomposelli JJ, Pomfret EA, Moses MA (2006) Matrix metalloproteinases (MMPs) activity after live donor adult liver transplantation (LDALT). *Am J Transplant* 6: 845.

Vu TH, Werb Z (2000) Matrix metalloproteinases: Effectors of development and normal physiology. *Genes Dev* 14: 2123-2133.

Xu J, Mingyao L, Post M (1999) Differential regulation of extracellular matrix molecules by mechanical strain of fetal lung cells. *Am J Physiol Lung Cell Mol Physiol* 276: L728-L735.

Yang EV, Gardiner DM, Carlson MRJ, Nugas CA, Bryant SV (1999) Expression of MMP-9 and related matrix metalloproteinase genes during axolotl limb regeneration. *Dev Dyn* 216: 2-9.