

# **REPAIR AND REGENERATION OF THE PRONEPHRIC KIDNEY IN *XENOPUS LAEVIS* TADPOLES**

A dissertation

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## ABSTRACT

The kidney is a unique organ in that it develops through three distinct forms of increasing complexity, with the most embryonic form (pronephros) serving as the functional organ in the tadpoles of the amphibian model system, *Xenopus laevis*, and the most complex form (metanephros) being found in adult humans. Since the developmental programs that govern organogenesis in all three kidneys are conserved, and all kidneys share the same structural units and basic functions, we can utilize these simpler pronephric kidneys to investigate the repair mechanisms in induced renal damage. To accomplish this, we developed a novel pronephrectomy technique to excise specific parts of the kidney in *X. laevis* tadpoles, and examined the repair responses that followed. The results of these studies demonstrate for the first time that an amphibian pronephros is able to regenerate lost structures. We also begin to elucidate the mechanisms through which this regenerative phenomenon occurs, and have revealed dualistic roles for the extracellular matrix remodeler, Matrix metalloproteinase-9. This protease is expressed during two distinct windows of pronephric regeneration: immediately after injury, and again five days later. While the early expression of XMMP-9 promotes pronephric regeneration, proteolytic activity of this enzyme during the second phase appears to inhibit this regenerative process. In this dissertation, we propose likely mechanisms for these disparate roles of XMMP-9.

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## LIST OF ABBREVIATIONS

Ab.....	antibody
AEC.....	apical epidermal cap
AF488.....	Alexa Fluor 488
AKI.....	acute kidney injury
ANOVA.....	analysis of variance
AP.....	alkaline phosphatase
BCIP.....	5-bromo,4-chloro,3-indolylphosphate
BF.....	bright field (microscopy)
BSA.....	bovine serum albumin
CDC.....	Center for Disease Control and Prevention
Chrom.....	chromogenic solution
CKD.....	chronic kidney disease
DEPC.....	diethylpyrocarbonate
Dig.....	digoxigenin
Dmg Ctrl.....	damage control (tadpole)
DMSO.....	dimethyl sulfoxide
DPF.....	day(s) post-fertilization
DPN.....	day(s) post-nephrectomy
ECM.....	extracellular matrix
EDTA.....	ethylenediaminetetraacetic acid
EMT.....	epithelial-mesenchymal transition
ESRD.....	end-stage renal disease
EtOH.....	ethanol
H <sub>2</sub> O.....	water
H <sub>2</sub> O <sub>2</sub> .....	hydrogen peroxide
HI.....	heat-inactivated
HPN.....	hour(s) post-nephrectomy
Hyb.....	hybridization (buffer)
IACUC.....	Institutional Animal Care and Use Committee
IgG.....	Immunoglobulin G
IHC.....	immunohistochemistry
ISH.....	<i>in situ</i> hybridization
MeOH.....	methanol
MMP.....	matrix metalloproteinase
MMR.....	Marc's Modified Ringer's solution
MOMP.....	mitochondrial outer membrane permeabilization
MRL.....	Murphy Roths Large (mouse strain)
NBT.....	nitroblue tetrazolium
Neph.....	nephrectomized (tadpole)
NF.....	Nieuwkoop and Faber, 1994 (staging <i>Xenopus</i> )
NKF.....	National Kidney Foundation
NSF.....	National Science Foundation
O/N.....	overnight
PBS.....	phosphate buffered saline



PBTr ..... phosphate buffered saline with Tris  
 PFA..... paraformaldehyde  
 PK..... proteinase K  
 PTW..... phosphate buffered saline with Tween-20  
 PCD ..... programmed cell death  
 RT..... room temperature  
 Sib Ctrl..... sibling control (tadpole)  
 SSC..... saline sodium citrate buffer  
 SSPE..... saline sodium phosphate EDTA buffer  
 TBM ..... tubular basement membrane  
 TIMP1..... Tissue inhibitor of metalloproteinase 1  
 UNOS ..... United Network for Organ Sharing  
 UUO ..... unilateral ureteral obstruction  
 WH ..... wound healing  
 WIHC ..... whole-mount immunohistochemistry  
 WISH..... whole-mount *in situ* hybridization  
*X. laevis* ..... *Xenopus laevis*

**Repair and regeneration of the pronephric kidney**  
**in *Xenopus laevis* tadpoles**

# **CHAPTER ONE**

## **Introduction**

The kidney is a vital organ that performs essential homeostatic functions in animals, allowing them (and us) to survive and thrive in diverse environments. For this reason, several organisms, including both vertebrates and invertebrates, have developed reparative processes to counteract the detrimental effects of injuring or losing renal tissue. However, while these repair phenomena have been observed and studied for several decades, the mechanisms regulating them are still poorly understood. So far, the renal repair community has demonstrated that, unlike piscine (fish) and tadpole kidneys, mammalian kidneys have a limited capacity for restoring structural components after severe damage. However, all vertebrate kidneys share important characteristics (such as developmental programs, form and function) and thus investigating repair in these aquatic model systems is essential for elucidating the regenerative potential that may be intrinsic to all vertebrate renal cells. This dissertation contributes to this research field by (1) establishing the amphibian *Xenopus laevis* as a useful model for investigating regeneration of embryonic kidneys, (2) describing some of the cellular events that epitomize this repair, and (3) proposing likely mechanisms through which this regenerative process may be occurring.

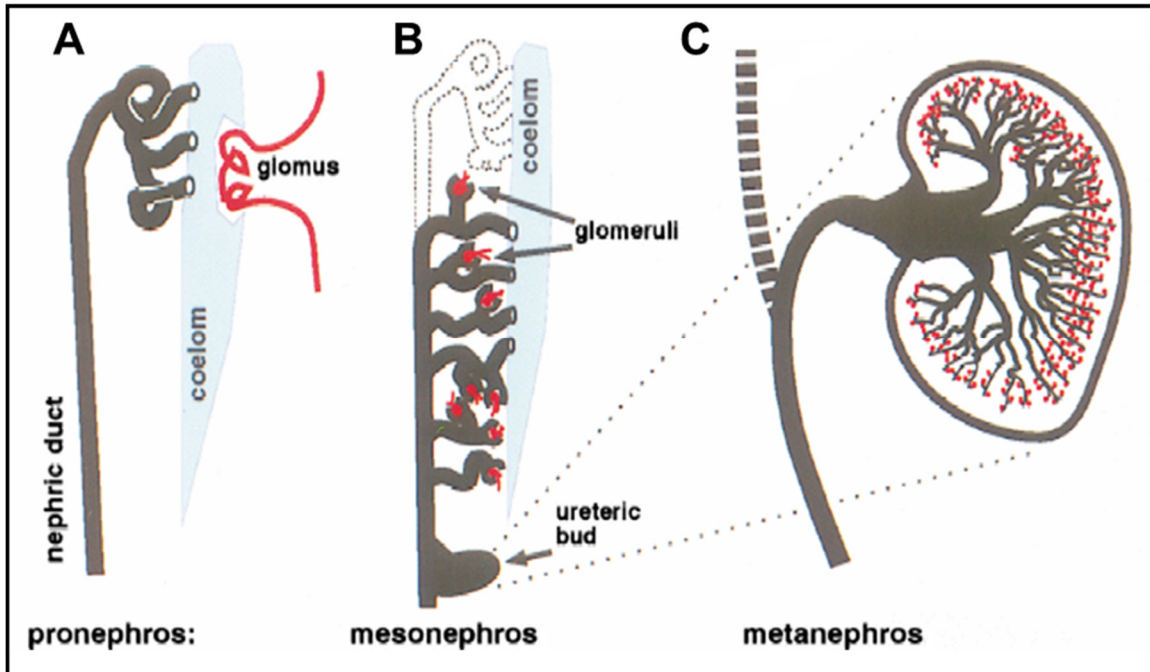
## **The Kidney**

### *Overview of vertebrate kidney development*

During embryogenesis in animals, organogenesis follows gastrulation (establishment of the three primary germ layers) to confer shape and function to developing organs. For most tissues, this process involves cellular differentiation and morphological rearrangements at the terminal anatomical site. Kidney development is

unique in that it occurs through a series of three distinct forms, the pronephros, the mesonephros, and the metanephros, which vary in location along the anterior-posterior axis at different stages of embryogenesis (Saxén, 1987). All three kidneys are derived from the intermediate mesoderm (Attia et al., 2012; Dressler, 2006; Vize et al., 1995) and develop in response to similar inductive signals between mesenchymal and epithelial tissues (Kuure et al., 2000; Carroll et al., 1999a; Vize et al., 1997). They develop as bilaterally symmetrical organ pairs and share the basic functional unit of all vertebrate kidneys, the nephron. However, these three kidneys vary greatly in nephron number and thus morphological complexity (Figure 1.1).

The pronephros develops first and is the most anterior of all kidneys (Vize et al., 1995). It is the functional kidney found in embryos and larvae of phylogenetically basal Gnathostomata (jawed vertebrates), including fish (Drummond and Davidson, 2010; Armstrong, 1932) and amphibians (Møbjerg et al., 2000; Vize et al., 1995; Fox, 1963; Jaffee, 1954; O'Connor, 1940; Howland, 1916), where it exists as a single nephron located on both sides of the animal (Carroll et al., 1999b; Drummond et al., 1998). It also functions as part of the adult excretory system in extant species of the primitive Agnathan class of (jawless) fish, such as lampreys and hagfish (Ellis and Youson, 1989). In more recently evolved taxa (reptiles, birds and mammals), the pronephros is functionally vestigial and exists for a very transient period during embryogenesis (Saxén, 1987; Bremer, 1916). Despite its impermanence, development of the pronephros is critical for the formation of the mesonephros, and ultimately, the metanephros (Jones, 2005; Vize et al., 1997; Vize et al., 1995; O'Connor, 1940).

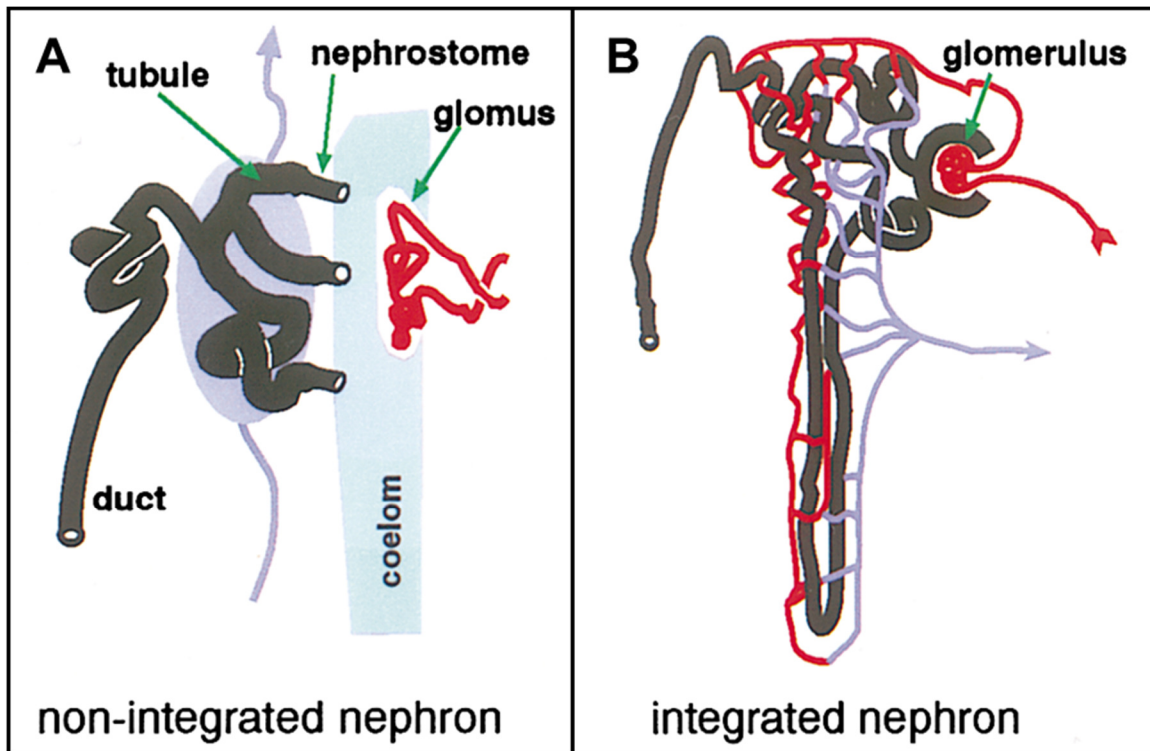


**Figure 1.1 Schematic of sequential kidney formation.** The first kidney to form is the pronephros (A), followed by the mesonephros (B) and ultimately the metanephros (C). Each sequential kidney is larger, more complex and develops more posteriorly to the previous form. All three kidneys consist of tubules and duct structures (dark grey) as well as a vascularized component (red). The coelomic cavity connects the pronephric glomus and some mesonephric glomeruli to their respective tubule networks. Image modified from Vize et al., 1997.

As Gnathostomata embryos and juveniles develop, the pronephros degenerates and all urinary and homeostatic functions are taken over by the mesonephros (Carroll et al., 1999a). Mesonephric kidneys are typically comprised of several nephrons (Vize et al., 1997; Kingsley, 1917) and develop caudally to the pronephros (Lipschutz, 1998). In adult anamniotes, the mesonephros functions as the primary excretory organ, and is then referred to as the opisthonephros (Fedorova et al., 2008; Barch et al., 1966). In contrast, mesonephroi in amniotes function briefly (a few days to many weeks depending on the organism) during mammalian intrauterine development (Moritz and Wintour, 1999; Tiedemann and Wettstein, 1980) or egg incubation (Doneen and Smith, 1982). The mesonephros then regresses (Pole et al., 2002) as the terminal metanephric kidney develops. The metanephros forms posterior to the mesonephros, is comprised of up to a million nephrons (Saxén, 1987), and is retained through adulthood (Lipschutz, 1998).

#### *Structure and function of the pronephric kidney*

The pronephros was first identified in the mid 1800's and has been described in detail in several sources (Vize et al., 1997). Compared to the multi-nephron networks of the mesonephric and metanephric kidneys, the architecture of the pronephros is relatively simple. The single nephron of the pronephric kidney is comprised of three main components: the pronephric corpuscle/glomus, the pronephric tubules and the pronephric duct (Brändli, 1999; Fox, 1963) (Figure 1.2). These segments are histologically, morphologically and functionally analogous to the nephrons in human metanephric kidneys, and as such the pronephros is a useful model for understanding both the developmental and reparative mechanisms in more complex kidneys.



**Figure 1.2 Schematic of nephron anatomy.** All nephrons contain tubules, a duct, and a glomus/glomerulus. In amphibian pronephroi (A), the glomal filtrate enters the coelom and is then wafted into the tubules via cilia in the nephrostomes. In metanephric nephron (B), glomeruli are fully integrated into the tubule network. Reabsorbed nutrients and water reenter the blood supply through the venous systems associated with the nephron (purple). Image modified from Vize et al., 1997.



The pronephros filters blood via the glomus, the vascularized portion of the pronephric corpuscle, which is supplied by branches of the dorsal aorta. Pronephric gloma are composed of fenestrated endothelial cells and podocytes (Drummond and Majumdar, 2003). In fish, the glomus is large and closely integrated with the nephric tubules and so is referred to as a glomerulus (Drummond et al., 1998). In amphibian pronephroi, the glomus connects to a cavity called the nephrocoele via the pronephric capsule (the non-vascular component of the pronephric corpuscle) (Brändli, 1999). This coelomic cavity, which is lined with squamous mesothelial cells (Wrobel and Süß, 2000), is also associated with the mesonephric glomeruli in fish, but is absent in mammalian metanephric kidneys (Figures 1.1 and 1.2)

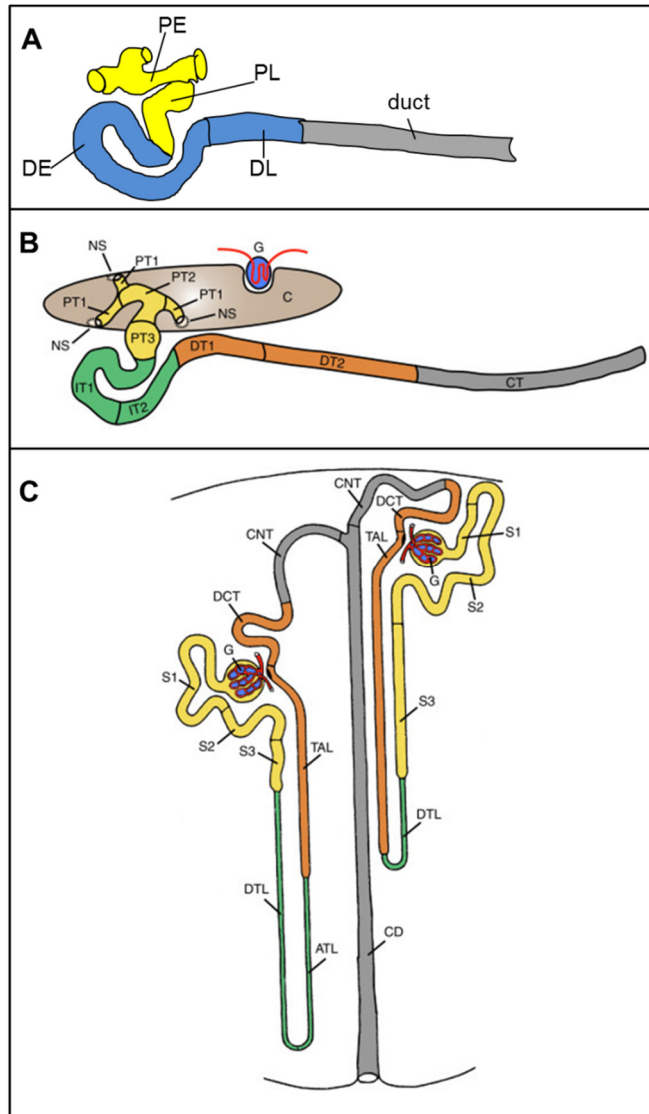
The glomal filtrate is swept forward by cilia lining the cuboidal epithelial cells of the nephrostomes. Each thin nephrostome tube funnels filtrate into the wider lumen of a branch of the proximal tubules, which are comprised of columnar epithelial cells with dense microvilli on the apical surface, and extensive convoluted lateral and basal membranes (Møbjerg et al., 2000) . This apical brush border is responsible for the majority of the osmoregulation functions (solute reabsorption and pH regulation) performed by the pronephric kidney (Christensen et al., 2008; Zhou and Vize, 2004). Resorbed nutrients, salts, and water are returned to the blood via the venous system surrounding the tubules (Vize et al., 1997) (Figure 1.2). The lateral and basal membranes may facilitate transepithelial transport as seen in the proximal tubules in mammals (Aronson, 1989). The number of nephrostomes, and corresponding number of proximal tubule branches, varies among organisms (Vize et al., 2003). In *Xenopus laevis* tadpoles, there are three nephrostomes connected to three proximal tubule branches (Brennan et al.,

1998). These dorso-anterior branches connect and empty filtrate into a common branch of the proximal tubules called the broad tubule, which adjoins the more posteriorly located distal tubules.

The cuboidal epithelial cells of the distal tubules lack an apical brush border but have an extensive basal membrane folding. Nitrogenous waste flows through the narrow lumen of the distal tubules and into the pronephric duct. The duct, otherwise known as the connecting tubule, is comprised of at least two distinct types of low cylindrical cells that lack apical microvilli and basal membrane folding (Møbjerg et al., 2000) and is responsible for carrying urine to the rectal diverticulum and ultimately to the cloaca (Drawbridge et al., 2003), where it is excreted.

#### *Segmentation of the pronephric tubules*

Although only two cell types are known to comprise the pronephric duct (Raciti et al., 2008), the pronephric tubules are more extensively segmented. Until recently, the proximal and distal tubules were divided into so called “early” (connected to the nephrostomes) and “late” (connected to the duct) regions (Zhou and Vize, 2004) (Figure 1.3). However, through large-scale gene expression mapping, Raciti and colleagues have provided the pronephric community with a new model for nephron organization that closely resembles the segmentation seen in mammalian kidneys (Raciti et al., 2008) (Figure 1.3). This model introduces the “intermediate tubule” segment, which expresses several kidney genes not found in the proximal and/or distal tubules that adjoin it on either side (Raciti et al., 2008).



**Figure 1.3 The evolution of nephron segmentation models.** A schematic of the model put forth by Zhou and Vize (2004) (A) designates early (E) and late (L) segments of the proximal (P, yellow) and distal (D, blue) tubules in the pronephric kidney. A new model introduced by Raciti et al. (2008) demonstrates that pronephric (B) and metanephric (C) nephrons are comprised of analogous segments, as indicated by the color coding in this schematic: yellow, proximal tubules (pronephric PT1, PT2, and PT3; metanephric segments S1, S2, and S3); green, intermediate tubules (pronephric IT1 and IT2; metanephric descending thin limb [DTL] and ascending thin limb [ATL]); orange, distal tubules (pronephric DT1 and DT2; metanephric thick ascending limb [TAL] and distal convoluted tubule [DCT]); and grey, connecting tubules (pronephric CT; metanephric connecting tubule [CNT] and collecting duct [CD]). Nephrostomes (NS) and coelom (C, brown) are also present in the pronephros. Image modified from Raciti et al., 2008.

While this hugely impressive undertaking has provided us with an updated nephron model and many new renal gene expression profiles, it has also expanded the way we must think about kidney repair. As will be discussed later in greater detail, damaged renal tubules in several injury models are often replaced by surviving tubule cells. Identifying the source of these cells in any renal repair or regenerative pathway is paramount to determining the mechanisms involved. Importantly, the work described in this thesis specifically focuses on characterizing regeneration of pronephric proximal tubules, and thus a keen understanding of tubule anatomy is critical to this endeavor.

### **Animal Models of Kidney Repair**

Several vertebrate models of kidney disease have been used to investigate the reparability of nephric structures, most often after damage to the tubular or glomerular basement membrane. Mammalian metanephric kidneys undergo epithelial replacement, with adjacent surviving cells migrating and proliferating to repopulate the denuded basement membrane of injured nephrons (Bonventre, 2003; Nony and Schnellmann, 2003; Gobe and Buttyan, 2002; Gobé et al., 1995; Nonclercq et al., 1992; Cuppage et al., 1972; Cuppage and Tate, 1967). Compensatory hypertrophism also occurs in both the injured kidney (Wesson, 1989) and the contralateral kidney (Menè et al., 2003; Sheridan and Bonventre, 2000).

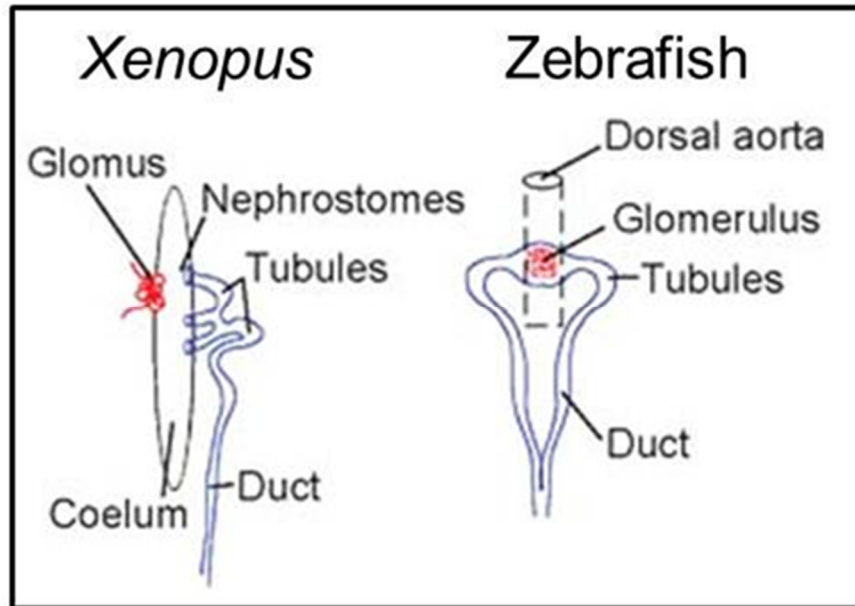
Neonephrogenesis, however, has only been observed in several species of fish (Davidson, 2011). After acute injury to proximal mesonephric tubules, teleost fish display a repair process similar to mammals with local proliferation of epithelial cells reestablishing tubule integrity (Salice et al., 2001; Reimschuessel and Williams, 1995).

However, both teleost fish (bony skeletons) and elasmobranch fish (cartilaginous skeletons, e.g. sharks and rays) also possess the unique ability to initiate *de novo* nephrogenesis after chemotoxic mesonephric injury. This phenomenon has been observed in the skate *Leucoraja erinacea* (Elger et al., 2003), as well as zebrafish (Diep et al., 2011), goldfish, *Carassius auratus* (Liu et al., 2002; Salice et al., 2001; Reimschuessel and Williams, 1995), catfish, rainbow trout, tilapia, toadfish, and tom cod (Reimschuessel, 2001). While these fish studies have contributed immensely to our understanding of the repair response of mesonephroi to chemical-induced nephrotoxicity, the fish system is less conducive to mechanical injury and as such is less well-represented in the current literature (Elger et al., 2003). Partial pronephrectomy on fish is complicated by the presence of a fused glomus, shared by both kidneys (Drummond, 2000) (Figure 1.4). In contrast, amphibian pronephroi are easily accessible and tadpoles are amenable to surgical manipulation.

In the late 1960's, biologists revealed the propensity for *X. laevis* to undergo contralateral hypertrophy in response to unilateral partial pronephrectomy (Chopra and Simnett, 1971; Chopra and Simnett, 1970; Chopra and Simnett, 1969; Simnett and Chopra, 1969), however no studies have examined the cellular and molecular responses at the ipsilateral (operated) site of renal injury. The results presented in this dissertation contribute the first evidence of ipsilateral tubule regeneration after nephrectomy in the *X. laevis* pronephros.

#### *Xenopus laevis as a model for kidney repair*

There is considerable interspecies variation among regenerative capacities, with



**Figure 1.4 Schematic comparing the pronephric kidneys of *Xenopus laevis* and *Danio rerio* (zebrafish).** The glomus in the amphibian pronephric kidney is non-integrated and exists in pairs (one on each side supporting a single pronephros). The zebrafish pronephroi pair, however, both integrate with a singular glomerular unit. Image modified from Dressler, 2006.

invertebrates typically exhibiting superior proficiency to vertebrates (Knapp and Tanaka, 2012; Sánchez Alvarado, 2000; Monks, 1904; Monks, 1903). Among vertebrates, however, members of the Amphibia class are unique in their ability to regenerate several larval and adult tissues. For this reason, both the Urodela (newts and salamanders) and Anura (frogs and toads) orders have produced a number of popular model organisms used in regenerative studies (Tsonis, 2000).

*Xenopus laevis* has long been a favorite among laboratory research animals due to its experimental tractability, rapid speed of embryonic and larval development, and adaptability to laboratory conditions (relatively easy to culture embryos, raise juveniles and care for adults). In addition, unlike in mammalian systems, *Xenopus* embryos and larvae develop independently of the mother and as such are readily available for invasive manipulations, such as the unilateral pronephrectomy performed in the studies described herein. Additionally, *Xenopus* has been a popular study system for determining the molecular mechanisms that regulate kidney development (Wessely and Tran, 2011; Jones, 2005; Hensey et al., 2002; Brändli, 1999; Carroll et al., 1999b; Brennan et al., 1998; Vize et al., 1997). This popularity provides a huge benefit for studying repair mechanisms in *Xenopus*, as renal regeneration may recapitulate these developmental programs.

Unfortunately, the pseudotetraploid genome of *Xenopus* has caused a considerable delay in the development of genetic tools, unlike those currently available for studying another well-established non-mammalian model system, the zebrafish (Poss et al., 2003). However, recent advances in the creation of a chemical genetic screening system (Tomlinson et al., 2012; Wheeler and Liu, 2012; Wheeler and Brändli, 2009) may expand

and/or provide new avenues of research with *Xenopus*. Importantly, the *Xenopus* and mammalian genomes are surprisingly similar (Hellsten et al., 2010), which may account for the comparable regenerative capacities observed in these two systems (Gargioli and Slack, 2004; Slack et al., 2004). These advantages, among several others (Beck et al., 2009), make the *Xenopus* system a uniquely suitable model organism to study renal regeneration.

## **Regeneration**

Regeneration as a field of study has been an active area of inquiry for more than two and a half centuries, with the first published work describing a regenerative event being written by the Swiss naturalist Abraham Trembley (1710-1784). In his 1744 book entitled *Memoires pour servir a l'histoire d'un genre de polypes d'eau douce, a bras en forme de cornes* (*Memoirs concerning the natural history of a type of fresh-water polyp, with arms shaped like horns*), Trembley describes the cephalic regeneration of a microscopic fresh-water animal, which he named *Hydra*, after the nine-headed water-serpent of Greek mythology. Since then, countless studies have continued to explore the cellular, molecular, bioelectric, electrochemical, and mechanotransductive mechanisms that contribute to regeneration. While all organisms may possess the genetic information and cellular machinery required for regeneration, there is a disparity among phyla in the capacity to regenerate. This has been attributed to several factors, including the differential expression of regeneration inducers and effectors, the evolution of the adaptive immunity in jawed vertebrates, and the plasticity of cells that may contribute to



a regenerate. These three variables are particularly relevant to the research described in this dissertation and will be discussed below (and in future chapters) in greater detail.

As new information on the parameters that dictate successful regenerative events become available, researchers continue to redefine and categorize these phenomena. This classification system allows biologists to identify patterns that characterize specific types of repair, which provides predictive and recapitulative potential that can be optimized in the field of regenerative medicine. Definitive categorization is also useful for tissue engineers who rely on regenerative cues to develop novel, highly specialized, artificial systems capable of tissue repair. Additionally, a comprehensive understanding of regeneration is necessary for determining the evolutionary contexts for the emergence of this phenomenon in general.

#### *Definition and categorization of regeneration*

Simply stated, regeneration is the ability to restore cells, tissues or organs in response to damage, disease, casting, autotomy, or fission. It can also describe the homeostatic turn-over of short-lived cell types, such as blood cells (Zon, 2008), skin (Martin, 1997), bone (Huang and Ogawa, 2010; Panetta et al., 2010), and intestinal epithelium (Barker et al., 2008; Ishizuya-Oka, 2007). Mechanistic commonalities among regenerative processes in disparate systems have been used to classify these events into four categories: epimorphosis, tissue regeneration, morphallaxis, and compensatory growth (Agata et al., 2007; Stoick-Cooper et al., 2007; Carlson, 1978; Donaldson and Mason, 1975) (Table 1.1).

Epimorphosis and tissue regeneration both describe processes focused on the

Table 1.1 Categorization of four types of regenerative events.

Type of regeneration	Major characteristic	Blastema	Proliferation	Hypertrophy	Cell Source	Notable examples
Epimorphosis	regrowth	yes	yes	limited	typically multipotent or pluripotent	<i>Xenopus</i> tail, limbs, fish fins, deer antlers, planaria
Tissue Regeneration	regrowth	no	yes	limited	typically unipotent	skeletal muscle, hearts, lens
Compensatory Growth	remodeling	no	yes	often	typically unipotent and limited to adjacent tissues	liver
Morphallaxis	remodeling	no	no	yes	typically pluripotent	<i>Hydra</i>

regrowth of injured tissue. Epimorphic regeneration occurs via cellular proliferation and differentiation, and is preceded by the formation of a blastema at the site of injury. Regeneration blastemata are cell masses comprised of undifferentiated multipotent or pluripotent cells. These cells may either originate from progenitor populations that pre-exist in the body, as seen in regeneration of planaria (Colitti et al., 2005; Price et al., 2005; Price and Allen, 2004; Reddien and Sánchez Alvarado, 2004) and deer antlers (Colitti et al., 2005; Price and Allen, 2004), or they may arise from the dedifferentiation of mature tissue at the site of injury, as seen in the regeneration of urodele jaws (Graver, 1978), and fish fins (Nakatani et al., 2007; Poss et al., 2003; Nechiporuk and Keating, 2002). Some epimorphic events, however, utilize both stem-like progenitor cells and dedifferentiated cells during tissue reconstruction. This has been observed in zebrafish heart regeneration (Lepilina et al., 2006) and during restoration of amputated appendages comprised of multiple tissues such as urodele limbs (Tweedell, 2010; Mochii et al., 2007; Gargioli and Slack, 2004; Kintner and Brockes, 1984), anuran tails (Mochii et al., 2007; Gargioli and Slack, 2004), and in the arm of the crinoid echinoderm *Antedon mediterranea* (Carnevali et al., 1995).

Tissue regeneration also features proliferation of undifferentiated cells, but this term is used to describe local and limited organ repair, often involving only one cell type (Stoick-Cooper et al., 2007), and does not include blastema formation. An example of this can be seen in vertebrate skeletal muscle during which resident satellite cells (quiescent progenitor cells which appose the basal lamina) proliferate and fuse after injury to regenerate syncytial muscle fibers (Chen et al., 2006; Morrison et al., 2006; Holterman and Rudnicki, 2005; Montarras et al., 2005; Slack et al., 2004; Zammit et al.,

2002; Mauro, 1961). Tissue regeneration is also epitomized by proliferation of neural stem cells in the anuran brain (Endo et al., 2007) and cardiomyocytes in vertebrate hearts (Senyo et al., 2013; Kikuchi and Poss, 2012; Poss et al., 2002; Oberpriller et al., 1995). Transdifferentiation (reprogramming of differentiated cells) during tissue regeneration has also been observed, and is exemplified by select ocular tissues. The vertebrate lens can be regenerated by either Wolffian transdifferentiation (dorsal pigmented iris epithelium to lens) (Imokawa and Brockes, 2003; Yamada, 1967) or cornea-lens transdifferentiation (Filoni et al., 1995; Overton, 1965; Freeman, 1963). Similarly, the neural retina can be regenerated via transdifferentiation of the retinal pigmented epithelium or ciliary marginal zone cells in many vertebrate embryos (Araki, 2007; Moshiri et al., 2004; Mitashov, 1996), as well as in adult urodeles and anurans (Yoshii et al., 2007; Keefe, 1973; Stone, 1950).

Morphallactic regeneration and compensatory growth (Table 1.1) do not feature the restoration of injured tissue by undifferentiated progenitor cells, but rather focus on the remodeling of undamaged portions of the original organ or body. These types of regenerative events restore the original function of the organ/body, but never restore the original form. The primary difference between these two classifications is that compensatory growth occurs via proliferation of surviving cells, while morphallaxis is characterized by a distinct lack of hyperplasia. Both morphallactic regeneration and compensatory growth, however, often require hypertrophy of pre-existing cells to sufficiently compensate for tissue loss.

Compensatory growth occurs in the absence of de-differentiation (no blastema formation), as contributing cells divide while retaining their differentiated state. The

most popular model for compensatory growth is the vertebrate liver, which can be restored to functional capacity and original mass from as little as 25% of the initial tissue (Fausto, 2000; Higgins and Anderson, 1931). Liver regeneration involves both cellular proliferation and compensatory hypertrophy (Miyaoka et al., 2012). In the Anthozoan Cnidarian starlet sea anemone (*Nematostella vectensis*), oral regeneration also involves, and indeed requires cellular proliferation in both ectodermal and endodermal layers (Passamanek and Martindale, 2012).

The most examined model of proliferation-free morphallactic regeneration has been the fresh-water Hydrozoan Cnidarian *Hydra*, which can regenerate from fragments that are only 5% of the adult size (Bode, 2003; Shimizu et al., 1993). The dramatic cellular re-patterning of each fragment involves metaplastic transformation of select cell types (Siebert et al., 2008), which produces complete, albeit smaller, individuals with highly regulated head:body proportions (Bode and Bode, 1980). More current studies, however, have shown that tissue renewal in *Hydra* may involve cellular proliferation (Chera et al., 2009; Miljkovic-Licina et al., 2007), even though this event is not always critical for successful regeneration (Cummings and Bode, 1984; Hicklin and Wolpert, 1973). These recent findings in *Hydra* illustrate the need to continually update the categorization of regenerative processes.

Based solely on the findings obtained during the course of my research project, it would be premature to say definitively which type of regeneration is occurring in the pronephric kidney in *Xenopus laevis*. However, given the absence of typical blastema morphology observed here and in other studies, it is unlikely that proximal tubule regeneration occurs via a canonical epimorphic pathway. Additionally, the presence of

proliferation observed in most renal repair models (including some preliminary evidence in our studies), eliminates morphallaxis as a prospective mechanism.

While fish mesonephric kidneys are capable of *de novo* regeneration (Diep et al., 2011; Watanabe et al., 2009; Elger et al., 2003; Liu et al., 2002), and thus fall into the tissue regeneration category, it is still unclear whether amphibian pronephric proximal tubules repair through a similar pathway, or whether they utilize compensatory growth of surviving cells. It is also possible that amphibian pronephroi could regenerate through a combination of these two mechanisms. Interestingly, the propensity of mammalian proximal tubule cells to proliferate and re-epithelialize denuded tubular basement membranes (TBMs) (Liu et al., 2002; Reimschuessel, 2001) suggests that under permissive conditions, all vertebrate proximal tubules may possess the potential for regeneration. Thus, elucidating the cellular and molecular regulators that may create this type of permissive repair microenvironment will be valuable for confirming this hypothesis.

#### *Wound healing prepares the regenerative microenvironment*

In response to tissue damage, regeneration typically progresses through three sequential stages: (1) an immediate wound healing (WH) event, (2) a temporary accumulation of undifferentiated cells, and (3) cell differentiation, tissue patterning and restoration of function. The initial wound repair that precedes regeneration is a dynamic process that occurs through a series of four phases: hemostasis, inflammation, proliferation, and maturation. These steps allow the body to assess the damage and, under non-pathological conditions, prepare the wound environment for appropriate levels

of repair. WH has been extensively studied and reviewed in the literature (Murawala et al., 2012; Reinke and Sorg, 2012; Martin and Leibovich, 2005; Harty et al., 2003; Li et al., 2001; Witte and Barbul, 1997; Kirsner and Eaglstein, 1993). While the specifics of each event vary with species, developmental stage and tissue type, the mechanisms that define this phenomenon appear to be well conserved. An overview of the typical wound healing process will be briefly described below.

Immediately after injury, a cascade of events is initiated which constrict blood vessels to minimize blood loss and bacterial exposure. This initial hemostatic response (first phase of WH) includes the activation of platelets by thrombin to convert fibrinogen to fibrin, a key component of clots. Platelets also release growth factors that act as pro-inflammatory chemotactic signals to promote the repair cascade. Cells of the innate immune system, such as neutrophils, are recruited by these chemokines and initiate the inflammatory phase at the site of injury (second phase of WH). Monocytes differentiate into macrophages, and partner with neutrophils to phagocytize microbes as well as cellular debris and extracellular matrix (ECM) components. Mast cells and other mediators of inflammation are also recruited, but arrive later in the wound healing process. These diverse immunomodulatory cell types release growth factors and cytokines at the wound site, which coordinate the supramolecular assembly of the ECM. Cytokines also amplify the inflammatory signal by recruiting more leukocytes. Macrophages also stimulate angiogenesis, a critical component of the proliferative phase (third phase of WH). Collagen deposition, granulation and epithelialization may also occur at this time. These cellular activities effect wound contraction and ultimately wound closure. They also prepare the environment for the fourth phase of wound

healing, tissue reconstruction. In non-regenerative systems, this results in scar formation or fibrosis. In regenerative systems, extensive ECM remodeling results in the creation of a permissive regenerative environment, which supports the restoration of lost tissues.

#### *Wound healing in regenerative and non-regenerative kidneys*

Every incidence of injury must be addressed by some degree of wound healing, with the inflammatory phase of repair largely determining whether an event will proceed toward regeneration or cicatrization (scar formation) (Harty et al., 2003). Both regenerative and non-regenerative wounds are closed by re-epithelialization, however non-regenerative tissues tend to experience excessive matrix deposition (Liu, 2011; Wynn, 2008; Raghow, 1994). The resulting scar, primarily comprised of collagen (Huang et al., 2011; King et al., 2003; Stichel, 1999), serves to reestablish the anatomical continuity of the tissue, but will never restore its original function. In contrast, wound healing during regenerative events involves re-epithelialization in the absence of fibroproliferation (Tanaka and Galliot, 2009). Consequently, successful regeneration is characterized by the scar-free restoration of functional tissues or organs, and in some circumstances, of entire body plans (Sánchez Alvarado and Tsonis, 2006; Tsonis, 2000).

The mammalian kidney often behaves as a non-regenerative organ as renal damage typically leads to fibrosis caused by chronic inflammation (Liu, 2011; Hewitt et al., 2008; Wynn, 2008), which is produced by dysregulated innate and adaptive immune responses (Wynn and Ramalingam, 2012). In contrast to the mammalian system, fibroproliferation has not been described in regeneration of fish mesonephroi (Diep et al., 2011; Watanabe et al., 2009; Elger et al., 2003) or *X. laevis* pronephroi (Caine and



McLaughlin, 2013), and was only briefly observed after newt mesonephrectomy (Scadding and Liversage, 1974). These phylogenetically basal members of the infraphylum Gnathostomata (jawed vertebrates; Phylum: Chordata, Subphylum: Vertebrata) have retained a more ancestral immunity (Mescher et al., 2007) and thus are more likely to have an attenuated inflammatory response to renal damage as compared to their mammalian counterparts. Rather than become fibrotic, damaged renal structures in fish (Elger et al., 2003; Liu et al., 2002; Salice et al., 2001; Augusto et al., 1996; Reimschuessel and Williams, 1995) and tadpoles (Caine and McLaughlin, 2013) tend to display a greater propensity for regeneration.

#### *Regeneration and the adaptive immune system*

Importantly, a remarkable regenerative capacity has also been described in several other organs in fish and amphibians (Sánchez Alvarado and Tsonis, 2006; Nye et al., 2003; Brockes and Kumar, 2002; Tsonis, 2000). These findings support a prevailing hypothesis in regenerative biology, which posits that the evolution of adaptive immunity resulted in the restriction of regenerative ability (Mescher and Neff, 2004). Further evidence of this is seen in invertebrates, which completely lack adaptive immunity but have outstanding regenerative capacities (Wenemoser et al., 2012; Scimone et al., 2011; Bode, 2003; Mito et al., 2002; Kobayashi et al., 1999; Carnevali et al., 1995; French and Domican, 1982; Bryant, 1971; Monks, 1904).

Adaptive immunity originated with jawed vertebrates (Flajnik and Du Pasquier, 2004), but is less evolved in anamniotes as compared to mammals (Menger et al., 2010; Robert and Cohen, 1998). Additionally, adaptive immunocompetence varies among

amphibians groups: between urodeles and anurans (Mescher and Neff, 2004), between larval and adult stages of anurans (Robert and Cohen, 1998), and between developmental stages in anuran larvae (Fukazawa et al., 2009). For this reason, amphibian model systems provide a unique opportunity to demonstrate the relationship between adaptive immunity and regenerative capacity.

Urodeles, which are immunodeficient compared to anurans (Mescher and Neff, 2004), retain the ability to regenerate through adulthood (Lévesque et al., 2005; Bettencourt-Dias et al., 2003; Imokawa and Brockes, 2003; Flink, 2002; Gardiner et al., 1999; Torok et al., 1998; Ghosh et al., 1996; Mescher, 1996; Simon et al., 1995; Ferretti et al., 1991; Thouveny et al., 1991; Graver, 1978), while anurans simultaneously develop adaptive immunity and lose regenerative capacity as they approach metamorphosis. This regenerative decline has been most clearly demonstrated in the froglet/frog limb (Suzuki et al., 2006; Muneoka et al., 1986; Thorton and Shields, 1945). Anuran tadpoles, however, have remarkable regenerative abilities (Endo et al., 2007; Ishizuya-Oka, 2007; Mochii et al., 2007; Tazaki et al., 2005; Gargioli and Slack, 2004; Beck et al., 2003; Endo et al., 2000; Yokoyama et al., 2000; Endo et al., 1997; Sakaguchi et al., 1997; Dent, 1962; Singer, 1951), and concomitantly rely primarily on their innate immunity throughout these larval stages (Mescher and Neff, 2004; Robert and Cohen, 1998).

Among anurans, *Xenopus laevis* (larvae and adults) remain the preferred non-mammalian model for studying the ontogeny and phylogeny of the immune system (Nedelkovska et al., 2013; Goyos and Robert, 2009; Robert and Ohta, 2009; Du Pasquier et al., 1989). In particular, *X. laevis* tadpoles also provide a useful system in which to investigate the relationship between immunity and regeneration. Tadpoles are typically

able to regenerate tails and hind limbs after amputation (Tseng and Levin, 2008; Mochii et al., 2007; Gargioli and Slack, 2004; Christen et al., 2003), however these structures are regeneration-incompetent during specific stages of development (Slack et al., 2004; Muneoka et al., 1986). High-throughput studies between regenerative and non-regenerative hind limb buds have indicated differential expression of several immunomodulatory genes (Grow et al., 2006; King et al., 2003). Similarly, tails amputated in refractory and regenerative (pre- and post-refractory) periods displayed distinct immune responses, including a loss of regenerative capacity as development of the T-cell population progressed (Fukazawa et al., 2009).

While these studies clearly demonstrate an intimate relationship between adaptive immunity and regeneration in tadpole limbs, it is not yet known if a similar pattern is found in visceral organs like the kidney. However, we can use this knowledge of the role of immunity in tadpole repair to inform our investigation on pronephric regeneration. Specifically, since the adaptive immunity is not active in *X. laevis* tadpoles until 12 days post-fertilization (dpf) (Tomlinson et al., 2008; Smith et al., 2002), we can assume that pronephrectomies performed at 2 dpf (as in our studies) will attract an inflammatory response dominated by innate immune cells. These cells are likely members of the phagocytic myeloid lineage, which include neutrophils and macrophages (Robert and Ohta, 2009; Smith et al., 2002). Macrophage recruitment, in particular, may be responsible for mediating early cellular events that are required for successful pronephric proximal tubule regeneration. Our preliminary data suggest that these cells may be producing the matrix metalloproteinase, *Xmmp-9*, during a very early window of

pronephric repair, and that the proteolytic activity of this ECM remodeler is crucial for tubule regeneration.

#### *The TBM and MMP-9 in kidney regeneration*

The kidney is a “basement membrane-rich” organ (Andrews et al., 2000) and thus requires proper regulation of the ECM components that both provide positional support for cells, and communicate via cell-matrix interactions. In metanephric renal repair, the presence of an intact tubular basement membrane (TBM) largely dictates the ability of tubule cells to recover from damage (Wang et al., 2010; Yang et al., 2002). The TBM is primarily comprised of collagen Type-IV (Nony et al., 2001; Abrahamson and Leardkamolkarn, 1991), which is specifically degraded within the basal lamina by MMP-9 (Singh et al., 2010). Taken together, these studies clearly indicate the need for proper regulation of this enzyme to promote maintenance and restoration of the kidney TBM. After renal injury, MMP-9 could accomplish this goal by preventing excessive Collagen IV deposition and thus suppressing fibrosis in the kidney (Liu et al., 2009).

However, in the absence of adequate regulation, MMP-9 could potentially destroy healthy ECM and disrupt the TBM. Under these conditions, tubule cells can lose adhesion to the ECM and slough off into the lumen, potentially forming casts and causing further tubular obstruction (Nony et al., 2001). Additionally, proximal tubule cells that detach from the TBM due to disruptions in the Collagen IV matrix (Chromek et al., 2003) can enter an epithelial-to-mesenchymal (EMT) transition in which they are converted to myofibroblasts (Wang et al., 2010; Yang et al., 2002). This type of chronic TBM destruction is irreversible and ultimately leads to renal scarring and eventually end-stage

renal disease (ESRD) (Wang et al., 2010). Combined, these studies emphasize the importance of regulating MMP-9, as well as other cellular and molecular remodelers of the ECM during the inflammatory phase of repair.

#### *Potential cellular sources in kidney regeneration*

The chemical signaling and structural scaffolds established during inflammation and wound healing influence the re-patterning of tissues during regeneration. With few exceptions (such as morphallaxis and metaplasia), regeneration typically requires a source of undifferentiated cells in order to rebuild damaged tissues. This source may be attained by one (or a combination) of three mechanisms: local dedifferentiation of cells that survive the injury, dedifferentiation of undamaged neighboring cells that will contribute to the regenerate, and/or proliferation of a reserve progenitor cell population, which often retains some levels of oligo- or multipotency (Tanaka and Reddien, 2011).

In mammalian kidneys, surviving renal cells are the primary cellular source for repopulating damaged TBMs (Bonventre, 2003; Nony and Schnellmann, 2003; Gobe and Buttyan, 2002; Gobé et al., 1995; Nonclercq et al., 1992; Cuppage et al., 1972; Cuppage and Tate, 1967). In several injury models, the absence of surviving tubule cells has been shown to severely attenuate renal repair and diminish renal function (Humphreys et al., 2008; Kays and Schnellmann, 1995; Nadasdy et al., 1995; Witzgall et al., 1994; Wallin et al., 1992; Kovacs et al., 1982; Venkatachalam et al., 1978; Cuppage et al., 1972). Recently we have also shown that a complete loss of proximal tubules after nephrectomy is correlated with ablation of the regenerative capacity in the pronephric kidney (Caine and McLaughlin, 2013). However, it not unknown whether surviving pronephric tubule

cells dedifferentiate and revert to a more mesenchymal state prior to reconstruction of the tubule, as is seen in mice (Humphreys et al., 2006).

Alternatively, evidence from several studies in fish (Diep et al., 2011; Zeng and Hou, 2011) and mammals (Fuente Mora et al., 2012; Lusic et al., 2010; Kobayashi et al., 2008; Gupta et al., 2006; Haller et al., 2005; Kitamura et al., 2005; Lin et al., 2005; Morigi et al., 2004; Al-Awqati and Oliver, 2002) have provided evidence for a population of renal progenitor (stem-like) cells that reside in differentiated mesonephric and metanephric kidneys, respectively. It is possible that a similar cell population may also exist in the pronephric kidney, and may contribute to the proximal tubule regeneration observed in *X. laevis* tadpoles.

Regardless of whether cells contributing to the regenerate have dedifferentiated, transdifferentiated and/or migrated from a progenitor niche, ultimately they must be epigenetically programmed to reestablish the differentiated state of the regenerating tissue. These changes in transcript and protein expression profiles will subsequently define the cellular character of the regenerated structure, conferring cell size, shape, membrane potential, metabolic activity and responsiveness to extrinsic and intrinsic signals. Differentiation and patterning of the regenerate will thereby restore the structure and function of the original tissue.

## Summary of Chapters

The primary goals of this research were to determine if pronephric proximal tubules in *Xenopus laevis* tadpoles are able to regenerate after unilateral pronephrectomy, and to characterize the repair and/or regenerative processes that occur in response to severe tubule damage. In the following chapters, I discuss the progress we have made in accomplishing both objectives.

**Chapter 2** describes a novel unilateral nephrectomy technique that I developed to specifically excise proximal tubule segments from pronephric kidneys. In this chapter, we also provide the first evidence of regeneration of functional renal tissue in an amphibian system. Lastly, Chapter 2 describes the pattern of apoptosis and the expression of the matrix metalloproteinase *Xmmp-9*, observed during the first week after mechanical renal damage. Our results suggest that both apoptosis and *Xmmp-9* are involved in the wound healing/inflammatory response that occurs during the first day post-nephrectomy (0-1 dpn), and that *Xmmp-9* may have a regeneration-specific role from 5-7 dpn. This work has been published in *Developmental Dynamics* (Caine and McLaughlin, 2013).

**Chapter 3** examines the roles of apoptosis and XMMP-9 activity during the 0-1 dpn period. We performed loss-of-function assays for both apoptosis and XMMP-9 activity by exposing nephrectomized tadpoles to pharmacological inhibitors from 0-1 dpn, then assessed the regenerative success of exposed vs. unexposed/vehicle-exposed tadpoles at 21 dpn. Our results indicate that inhibition of the apoptotic event observed during the first 24 hours post-nephrectomy (hpn) correlates to greater regenerative success of renal tubules, suggesting that early cell death may be detrimental for

regeneration. We also demonstrate that XMMP-9 activity within 24 hours of renal damage promotes renal tissue regeneration. Chapter 3 is in preparation for submission (Caine et al., *in preparation*).

**Chapter 4** provides preliminary evidence for an inhibitory role of XMMP-9 activity from 5-7 dpn, and speculates on the mechanism through which this enzyme may be functioning. Based on the spatial expression pattern of *Xmmp-9* at 5-7 dpn (Chapter 2), it appears that XMMP-9-secreting cells are recruited to the site of tubule regeneration, where excessive proteolytic activity may be inhibiting the regenerative capacity of pronephric proximal tubules. This hypothesis is supported by the preliminary data obtained in “double inhibitor” experiments, whereby XMMP-9 activity was blocked from 0-1 dpn and then again at 5-7 dpn. The results of these double exposures suggest dualistic roles for this metalloproteinase in pronephric repair, and that loss of harmful XMMP-9 activity at 5-7 dpn is sufficient to compensate for the loss of beneficial XMMP-9 activity at 0-1 dpn.



## CHAPTER TWO

### **Regeneration of functional pronephric proximal tubules after partial nephrectomy in *Xenopus laevis***

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Running title: Tubule regeneration after nephrectomy

Key words: pronephros, kidney regeneration, renal repair, wound healing, Mmp-9, apoptosis, *Xenopus laevis*

Key Findings:

- Novel finding that *Xenopus laevis* pronephric kidneys are capable of regenerating functional proximal tubules within three weeks of nephrectomy.
- Apoptosis occurs within hours of nephrectomy. This event is not a general response to injury, but rather coincides with tubule excision.
- *Xmmp-9* is expressed in a biphasic pattern during the first week after removal of the proximal pronephric tubules. The timing of the first phase of expression is consistent with facilitating the wound healing process, whereas the second phase may be involved in the initial steps of tubule regeneration.

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Kidney repair/regeneration has been an active area of research for decades however the mechanisms regulating these processes are not well-understood. Additionally, while repair events in mesonephric and metanephric kidneys have been extensively studied (reviewed in Davidson, 2011), very little is known about the regenerative capacity of the pronephric kidney. Previous work on amphibian pronephroi has focused on the compensatory hypertrophy response of the unoperated, contralateral kidney after unilateral nephrectomy (Chopra and Simnett, 1970; Chopra and Simnett, 1969; Swingle, 1919; Howland, 1916). However, this research examines the repair response on the operated, ipsilateral side, and provides the first evidence of pronephric regeneration in *Xenopus*. We also examine apoptosis and *Xmmp-9* expression during the first week after nephrectomy.

## **ABSTRACT**

Background: While the renal system is critical for maintaining homeostatic equilibrium within the body, it is also susceptible to various kinds of damage. Tubule dysfunction in particular contributes to acute renal injury and chronic kidney disease in millions of patients worldwide. Since current treatments are highly invasive and often unavailable, gaining a better understanding of the regenerative capacity of renal structures is vital. Although the effects of various types of acute damage have been previously studied, the ability of the excretory system to repair itself after dramatic tissue loss due to mechanical damage is less well characterized. Results: A novel unilateral nephrectomy technique was developed to excise pronephric proximal tubules from *Xenopus laevis* tadpoles to

study tubule repair after injury. Immunohistochemical detection of protein expression and renal uptake assays demonstrated that *X. laevis* larvae have the capacity to regenerate functional proximal tubules following resection. Conclusions: We have validated the renal identity of the restored tubules and demonstrated their ability to function normally providing the first evidence of regeneration of renal tissue in an amphibian system. Importantly, this tubule restoration occurs via a process involving an early apoptotic event and the biphasic expression of the matrix metalloproteinase, *Xmmp-9*.

## INTRODUCTION

Because the kidney is necessary for the maintenance of vertebrate homeostasis, many organisms possess the ability to repair minor damage to nephric structures (Humphreys, 2011; Bonventre, 2010; Menè et al., 2003; Nony and Schnellmann, 2003). In spite of this, injuries that result in a reduction of nephron number in mammals, lead to a permanent renal deficit, as they are unable to replace lost or severely damaged nephrons. In contrast, some vertebrates, most notably fish, are able to undergo neonephrogenesis as adults, replacing damaged nephric tissue throughout their lifetime (Zhou et al., 2010; Watanabe et al., 2009; Elger et al., 2003; Salice et al., 2001; Augusto et al., 1996; Reimschuessel and Williams, 1995). Although the renal systems of vertebrates differ in complexity (metanephroi in mammals, reptiles and birds; mesonephroi in adult amphibians and fish; pronephroi in juvenile amphibians and fish) (Saxén, 1987), comparative studies provide unique opportunities to identify and

characterize the underlying mechanisms that facilitate the restoration of a severely damaged excretory system.

Even though studies have demonstrated that the mammalian metanephric kidney has remarkable regenerative capacity after experiencing acute ischemic and/or toxic injury, this repair is primarily restricted to repopulation of the denuded basement membrane of injured nephrons via local proliferation and migration of adjacent surviving epithelial cells (Bonventre, 2003; Nony and Schnellmann, 2003; Nonclercq et al., 1992). Compensatory hypertrophism also occurs in both the injured kidney (Wesson, 1989), and the contralateral kidney (Menè et al., 2003; Sheridan and Bonventre, 2000). In contrast, teleost (Liu et al., 2002; Reimschuessel, 2001; Salice et al., 2001; Reimschuessel and Williams, 1995) and elasmobranch fish (Elger et al., 2003) have demonstrated a greater capacity to restore damaged renal structures. Additional research has yielded important information regarding repair processes that occur after acute damage resulting from exposure to nephrotoxic agents, such as the aminoglycoside antibiotic, gentamicin (Diep et al., 2011; Watanabe et al., 2009; Hentschel et al., 2005; Augusto et al., 1996). However, while these chemotoxic studies have been extensively investigated, very little is known about the renal response to mechanical injury.

The ability to study the renal repair process after severe damage, such as that seen in patients with acute kidney injury (AKI) or chronic kidney disease (CKD), has proven to be more challenging. While these disorders are diagnosed based on loss of renal function, oftentimes the conditions are the result of obstructive injury and/or irreversible destruction of nephric tissue integrity. Experimentally inducing damage of this magnitude in metanephric and mesonephric kidneys can be difficult due to their

complexity and inaccessibility within the animal. The embryonic structure, the pronephros, provides a simpler and more easily accessible organ system with which to conduct these types of studies. Although laser ablation of pronephric tubules has recently been optimized in zebrafish as an alternative to chemotoxic damage (Johnson et al., 2011), no other mechanical injury models in pronephric kidneys currently exist. With this study, we offer a novel technique for inducing mechanical damage in renal tissue in the amphibian model organism, *Xenopus laevis*. While excision of fish pronephroi is complicated by a fused glomus, the tadpole has a separate glomus for each pronephros, enabling the partial or complete excision of one kidney. Additionally, the tadpole pronephros is located dorso-laterally, just beneath the skin, making it accessible for this type of manipulation. Furthermore, pronephric development is very well characterized (Brändli, 1999; Carroll et al., 1999b; Carroll et al., 1999a; Vize et al., 1997), and as such may provide useful insight into repair mechanisms in differentiated tissue. Although aspects of kidney repair have been previously studied in mammals (Imgrund et al., 1999; Nonclercq et al., 1992; Boti et al., 1982) and most recently in fish (Diep et al., 2011; Zhou et al., 2010; Watanabe et al., 2009) little is known about the underlying mechanisms that mediate these events.

In this study, we provide the first evidence of pronephric kidney regeneration after severe mechanical damage in an amphibian system. Additionally, our initial characterization of the cellular and molecular basis of renal repair revealed both an early apoptotic event that occurs within hours of renal injury as well as a biphasic, regeneration-specific pattern of *matrix metalloproteinase-9* (*Xmmp-9*) expression. Moving forward, a greater understanding of events that facilitate tissue repair after

mechanical injury will be pivotal to help clarify whether similar processes can be re-established in mammalian metanephric renal systems.

## EXPERIMENTAL PROCEDURES

### *Tadpoles*

All experiments conducted were performed in accordance with the Guide for Care and Use of Laboratory Animals and were approved via the Institutional Animal Care and Use Committee (IACUC) at Tufts University. Adult female *Xenopus laevis* frogs were induced to ovulate via chorionic gonadotropin hormone (Chorulon) injection. Adult male wild type *X. laevis* frogs were sacrificed via intraperitoneal injection with tricaine (MS-222, Acros Organics) and the testes removed and stored in 1X modified Barth's saline (pH 7.5). Eggs were fertilized *in vitro*, and resultant embryos were dejellied in a 2% cysteine solution (pH 8) and reared in 0.1X Marc's Modified Ringer's solution [MMR; 10 mM NaCl, 0.2 mM KCl, 0.1 mM CaCl, 0.2 mM MgCl<sub>2</sub>, 0.5 mM HEPES, 1  $\mu$ M EDTA, pH 7.4] (Kay and Peng, 1991) at 14-25 °C. 0.1X MMR solution was changed daily. Embryos and tadpoles were staged according to Nieuwkoop and Faber (NF, 1994). Feeding-stage tadpoles were fed Sera Micron powdered growth food.

### *Unilateral pronephrectomy*

NF stage 37/38 tadpoles (Nieuwkoop and Faber, 1994) were randomly assigned to one of three treatment groups: nephrectomies, damage controls, or unoperated sibling controls. Tadpoles in all groups were first individually anaesthetized with 0.04% tricaine in 0.1X

MMR. During nephrectomies, fine forceps were used to make a small lateral incision adjacent to the left gill flap, and to peel back the skin covering the pronephros. Proximal tubules were identified via light microscopy as a thin tubular network, and were promptly excised and rinsed off the forceps by submersion and gentle agitation in 0.1X MMR. Nephrectomized tadpoles were immediately transferred to fresh 0.1X MMR to recover from anesthesia. Damage control tadpoles were poked multiple times through the skin into the left-side renal region. Tadpoles were placed in 14°C incubators overnight. After 24 hours, tadpoles were transferred to 18°C where they were maintained for the entirety of each experiment. At the desired time-point post-surgery, tadpoles were euthanized with tricaine, fixed for one hour at room temperature in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO<sub>4</sub>, 3.7 % formaldehyde), rinsed in 1X phosphate buffered saline (PBS), and gradually dehydrated to 100% methanol for storage at -20°C.

#### *Immunohistochemistry (IHC)*

To examine localization of proteins, whole mount IHC was performed on tadpoles (NF stages 37-45 and decapitated/tail-amputated NF stages 48-50). Briefly, tadpoles were permeabilized by washes in PBTr (1X PBS with 0.1 % Triton and 2 mg/ml bovine serum albumin [BSA]), blocked with 20% heat-inactivated goat serum in PBTr, and incubated overnight with primary antibody. Tadpoles were then rinsed in PBTr for 4-5 hours, blocked and incubated overnight in either goat anti-mouse or goat anti-rabbit IgG secondary antibodies conjugated to either alkaline phosphatase (AP; 1:1500) or Alexa Fluor 555 (AF-555; 1:300; Invitrogen, Sigma, or SouthernBiotech). Protein expression was detected after 5 hours of PBTr washes, and incubation in chromogenic buffer (100

mM Tris, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, 0.1 % Tween-20) with 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium (1:600 BCIP/1:600 NBT; Roche Diagnostics) or via fluorescence. Tadpoles were then rinsed in 1X PBS, post-fixed in MEMFA for 1-3 hours at room temperature or overnight at 4°C, dehydrated to 100% methanol and stored at -20°C. The following tissue types/markers were assessed through IHC: proximal tubule/3G8 (gift from Elizabeth Jones), pronephric duct/Alpha 5 subunit of Na, K-ATPase (Developmental Studies Hybridoma Bank, University of Iowa), skeletal muscle/12/101 (Developmental Studies Hybridoma Bank, University of Iowa), and apoptotic cells/active Caspase 3 (Abcam and BD Biosciences Pharminogen).

### *Histology*

After IHC, dehydrated tadpoles were gradually transferred to ethanol through a series of washes of increasing gradient (70%, 90%, 100%) then rinsed twice in absolute ethanol. Specimens were cleared in three successive 20 minute xylene incubations and infiltrated twice in paraffin wax at 60°C for 45 minutes, then wax-embedded in molds overnight. 10 µm sections were cut with a Leica 2255 rotary microtome, and mounted on Superfrost Plus glass slides. Sections were dewaxed in a series of xylene washes, counterstained with eosin (Fisher Scientific) and mounted with Permount (Fisher Scientific). Sections were then examined with an Olympus BX40 light microscope and photographed using DP Controller software.



### *In situ hybridization (ISH)*

To examine localization of mRNA transcripts, whole mount ISH was performed on tadpoles (NF stages 37-45 and decapitated/tail-amputated NF stages 48-50). Tadpoles were hybridized with digoxigenin-labeled antisense riboprobes as described previously (Harland, 1991). mRNA expression was detected via an anti-digoxigenin antibody conjugated to AP (1:1500; Roche Diagnostics) and a blue BCIP/NBT precipitate. Tadpoles were post-fixed as described for IHC. The following transcripts were detected through ISH: *Xmmp-9* (Carinato et al., 2000) and *nephrin* (Barletta et al., 2003).

### *Analysis of gene expression*

All tadpoles were examined for specific protein or mRNA expression under light/fluorescent microscopy, and representative individuals were photographed with a Nikon SMZ1500 stereomicroscope and Spot Insight Color digital camera and software. Area of gene expression in ipsilaterally nephrectomized and damage control kidneys was qualitatively compared to respective contralateral kidneys for all proteins and transcripts. For analysis of apoptosis, the number of cells expressing active Caspase 3 in the nephric region of all tadpoles was counted. An increase in active Caspase 3 at the site of nephrectomy was determined by observing more Caspase-positive cells on the ipsilateral side relative to the contralateral side. Damage control tadpoles and unoperated tadpoles were scored using the same criteria.

### *Renal uptake assay*

BSA conjugated to Alexa Fluor 488 (BSA-AF488; 66 kDa; Molecular Probes), was injected into nephrectomized and unoperated sibling control tadpoles at 23 dpn as described previously (Zhou and Vize, 2004). All tadpoles were observed in real-time under light/fluorescent microscopy and representative individuals were photographed with a Nikon SMZ1500 stereomicroscope and Spot Insight Color digital camera and software prior to fixation.

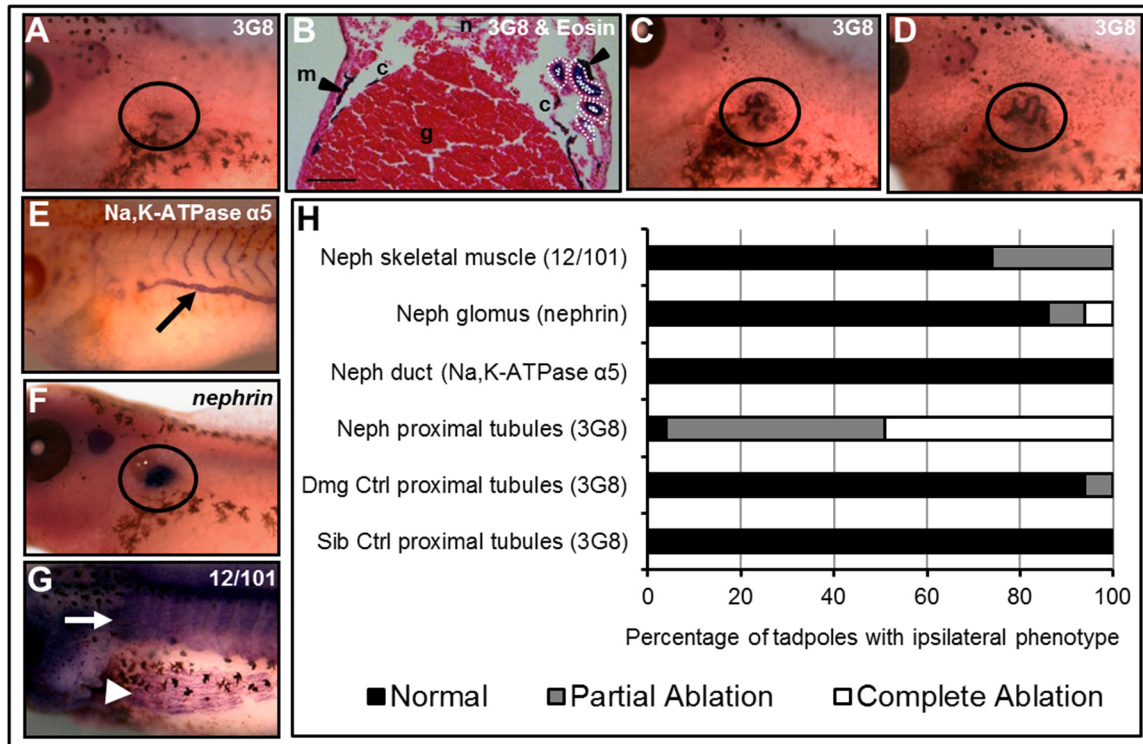
## **RESULTS**

### *Unilateral nephrectomy is a tractable and reproducible model of mechanical renal damage*

Pronephric kidneys in *X. laevis* are functional from 3 days post-fertilization (at room temperature, Nieuwkoop and Faber stage 37/38), and degenerate during pre-metamorphosis (beginning at NF stage 53), at which time the mesonephros starts to function (Nieuwkoop and Faber, 1994). In order to allow the maximum amount of time for the excised proximal tubules to regenerate, nephrectomies were performed at the onset of kidney function, three weeks prior to this natural degeneration process. NF stage 37/38 pronephric kidneys are cephalic organs located ventral to trunk somites I and II, in small opaque bundles on either side of the tadpole. The proximal tubules lie immediately adjacent to the skin, and can be identified via light microscopy as a thin tubular network once the skin has been pulled back. Careful excision with fine forceps was performed and excised tubules were visually identified upon removal.

In the majority of nephrectomized tadpoles, the proximal tubules were partially or completely removed as intended, as confirmed by the partial (Figure 2.1A) or complete (Figure 2.1B) ablation of 3G8 protein expression on the operated side (Figure 2.1H). The vast majority of damage control tadpoles retained undamaged proximal tubules, expressing the normal 3G8 pattern (Figure 2.1C and 2.1H). The 3G8 expression detected in the proximal tubules of unoperated sibling control tadpoles was always normal (Figures 2.1D and 2.1H).

Although nephrectomy undoubtedly disrupted the pronephric sinus (capillary system) surrounding the tubules, the surgery typically precluded damage to other nephric components as was demonstrated by examining non-tubule components of the pronephric kidney 1 day post-nephrectomy (dpn). Undamaged pronephric ducts, which express the alpha 5 subunit of the sodium-potassium adenosine triphosphate pump (Na, K-ATPase), were always observed at 1 dpn (Figures 2.1E and 2.1H). The glomus, which is situated medially to the tubules below trunk somites I and II, was undamaged in the majority of nephrectomized tadpoles, displaying normal expression of the podocyte marker, *nephrin* (Figures 2.1F and 2.1H). Additionally, the integrity of adjacent skeletal muscle was confirmed by examining 12/101 protein expression. Damage was rarely observed in either the somites or the hypaxial muscles, which flank the pronephros dorsally and ventrally, respectively (Figures 2.1G and 2.1H). These results demonstrate that unilateral nephrectomy can be used to create mechanical damage to a specific component of the pronephric kidney in *Xenopus laevis* tadpoles, while maintaining the integrity of neighboring tissues.

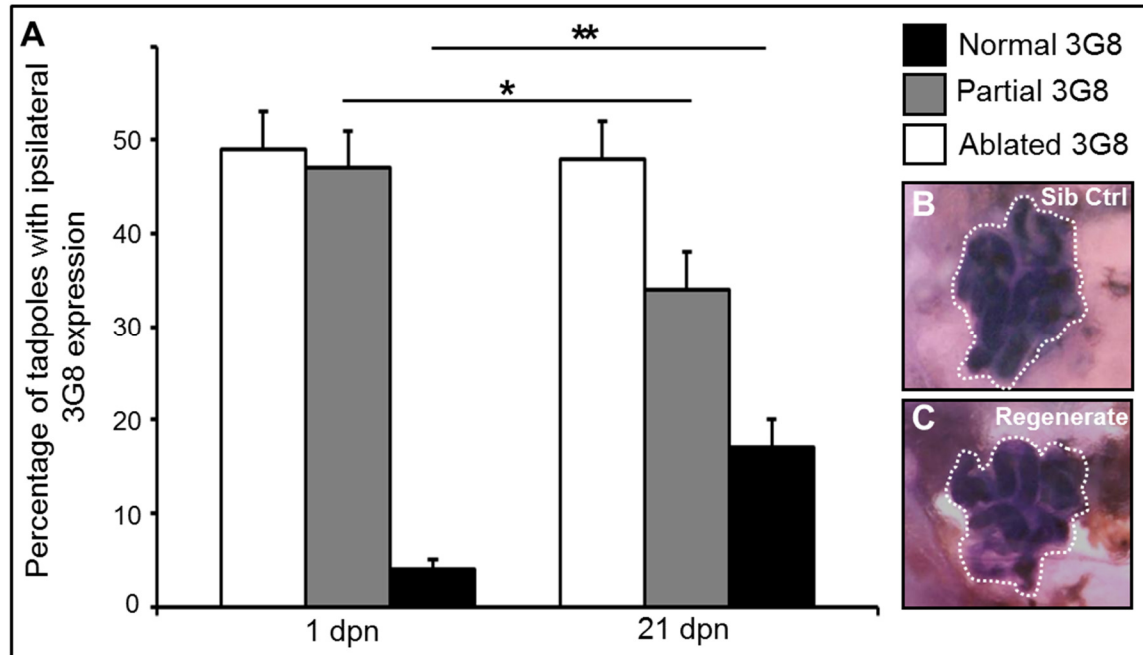


**Figure 2.1 Proximal tubules are successfully excised during partial nephrectomy in NF stage 37/38 *Xenopus laevis* tadpoles.** 3G8-positive tissue was often partially (A) or completely ablated (B; left side) at 1 day post-nephrectomy (dpn; 315 tadpoles), but occurred in the expected (phenotypically normal) pattern in the vast majority of damage control tadpoles (C; 52 tadpoles) and all unoperated sibling control tadpoles (D; 291 tadpoles). Damage in adjacent tissues was also examined via gene expression at 1 dpn. Normal expression of the alpha 5 subunit of Na, K-ATPase (E; 22 tadpoles), *nephrin* (F; 51 tadpoles), and 12/101 (G; 58 tadpoles) are retained in the pronephric duct, glomus, and skeletal muscle respectively, on the operated side of the majority of nephrectomized tadpoles (H). All whole-mount photographs are lateral views of representative tadpoles, with anterior to the left (A, C-G). Black circles highlight the renal area. Black arrow marks the pronephric duct. White arrow indicates somitic muscle and white arrowhead points to hypaxial muscle. Photograph of 10  $\mu$ m section of paraffin-embedded/eosin counter-stained 1 dpn tadpole (B), with 3G8-positive proximal tubules (broken white outlines) present on the unoperated contralateral side but absent on the operated side. Scale bar, 100  $\mu$ m; c, nephric coelom; g, gut; m, melanocytes (black arrowheads); n, notochord. Graph (H) illustrates the proportion of nephrectomized tadpoles with phenotypically normal, partially ablated, and completely ablated gene expression (in parentheses) in several tissues on the operated side.

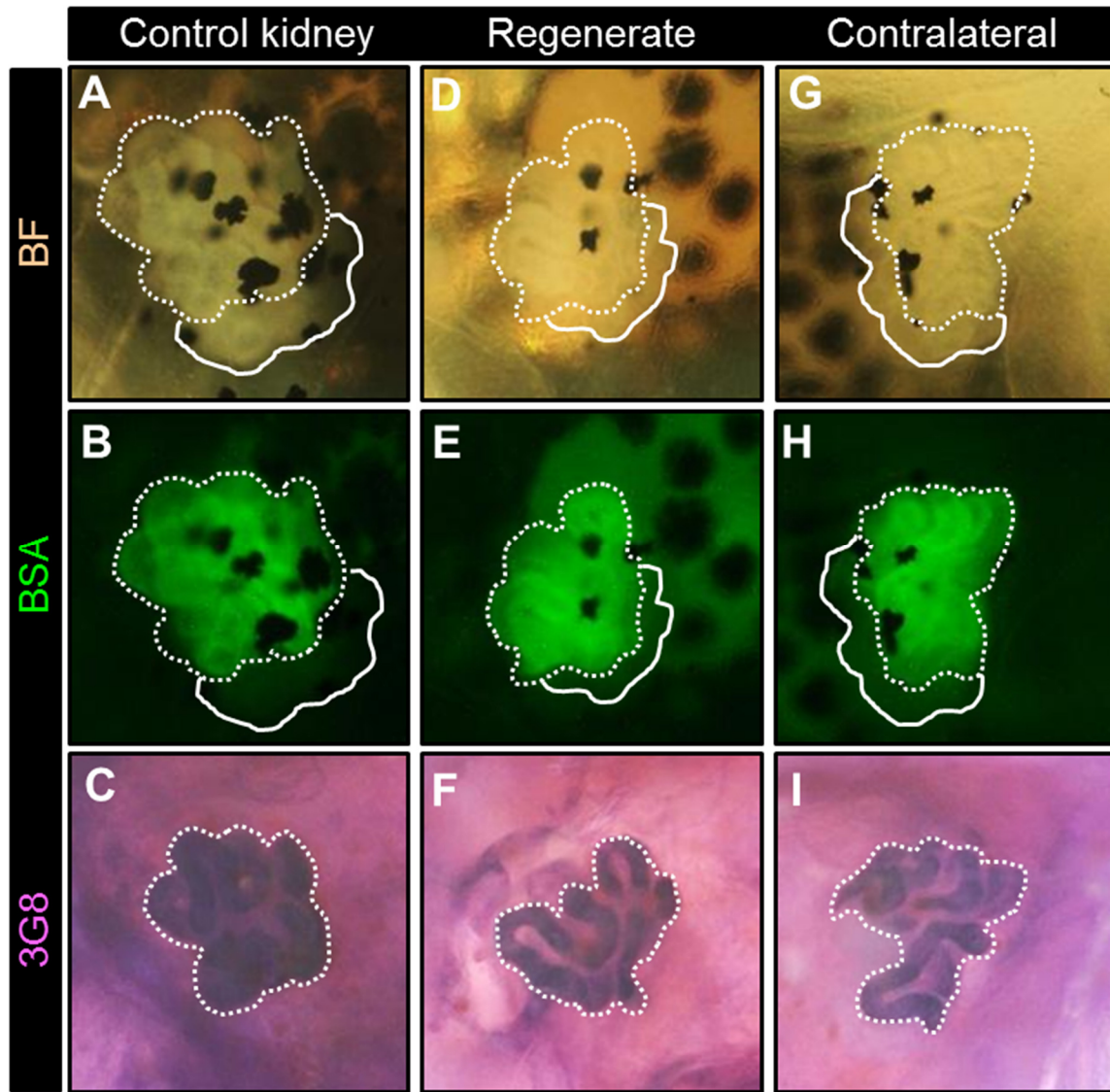
### *Functional proximal tubule tissue is restored after partial nephrectomy*

After establishing a technique that allows for the removal of a specific renal structure, the ability to restore proximal tubules was examined. The presence of differentiated proximal tubule tissue was confirmed via detection of 3G8 expression on the ipsilateral (operated) side of tadpoles at 1 dpn and 21 dpn, as well as in the kidneys of unoperated sibling controls (Figure 2.2). Only a few nephrectomized tadpoles (4%) retained normal 3G8 expression one day after nephrectomy (4%). In contrast, at 21 dpn, significantly more nephrectomized tadpoles possessed coiled proximal tubules expressing 3G8 normally on the operated side (17%,  $p = 0.00012$ , Student's T-test; Figure 2.2A). These regenerates were phenotypically comparable to unoperated control kidneys (compare Figures 2.2B and 2.2C). This demonstrates that a portion of the *X. laevis* tadpole population can successfully restore differentiated proximal tubules after extensive resection of this tissue. The remaining 21 dpn tadpoles either had no detectable 3G8-positive cells or had tubule tissue in varying stages of repair ("partial 3G8"; Figure 2.2A).

In order to determine if regenerated tubules regained renal function, we performed renal uptake assays at 23 days post-nephrectomy, as previously described (Zhou and Vize, 2004). Twenty-four hours after injection, the location of fluorescently tagged bovine serum albumin (BSA-AF488) was examined. All unoperated sibling control kidneys (Figures 2.3A-2.3B) and all contralateral kidneys in nephrectomized tadpoles (Figures 2.3G-2.3H), showed reabsorption of BSA into proximal tubule cells. Pronephric regenerates were also able to reabsorb BSA (Figure 2.3D-2.3E). As expected (Zhou and Vize, 2004), no fluorescence was observed in the distal tubules of any tadpoles (Figures 2.3B, 2.3E and 2.3H). All injected tadpoles were also examined for 3G8 protein



**Figure 2.2 *Xenopus laevis* tadpoles are able to regenerate proximal tubules 21 days after partial nephrectomy.** The majority of nephrectomized tadpoles showed partial or no 3G8 expression at 1 day post-nephrectomy (dpn). At 21 dpn, significantly more tadpoles expressed 3G8 normally in coiled proximal tubules on the operated side. Conversely, significantly fewer 21 dpn tadpoles displayed partial 3G8 expression. The proportion of 21 dpn tadpoles with no 3G8-positive ipsilateral tissue, is comparable to the percentage of the 1 dpn population with completely excised 3G8-positive tubules (A). 315 animals were assayed at 1 dpn. 458 animals were assayed at 21 dpn. Error bars indicate standard error among 19 replicates/group. Asterisks denote two-sample Student's t-test between 1 dpn and 21 dpn groups: \*,  $p=0.03$ ; \*\*,  $p=0.0001$ . All 21 dpn tadpoles with normal ipsilateral 3G8 expression in regenerated kidneys, displayed gene expression and tubule morphology similar to those observed in the unoperated sibling control tadpoles (compare B and C). Photographs are magnified lateral views of proximal tubules in representative tadpoles, with anterior to the left.



**Figure 2.3 Regenerated pronephric proximal tubules are functional three-weeks after excision.** All unoperated control kidneys (115 animals) were able to reabsorb fluorescently-tagged bovine serum albumin (BSA) into proximal tubules (A-B), as confirmed by the expression of the proximal tubule-specific protein, 3G8 (C). Of the 109 nephrectomized tadpoles assayed, 10 individuals (9%) restored ipsilateral coiled structures capable of both reabsorbing fluorescently-tagged-BSA (D-E) and expressing 3G8 (F). Comparable coiled morphology, BSA re-uptake capability and 3G8 expression were also seen in all unoperated contralateral proximal tubules (G-I). Photographs are lateral views of proximal tubules in representative tadpoles, with anterior to the left in A-F, and to the right in G-I. BF, bright field. Broken outlines indicate proximal tubules. Solid outlines indicate distal tubules.

expression to confirm proximal tubule identity of the regenerates (compare Figures 2.3C, 2.3F and 2.3I). These results demonstrate that three weeks after severe mechanical injury, restored proximal tubules are able to function normally, contributing to homeostatic equilibrium of the tadpoles. This provides the first evidence of regeneration of functional nephric structures in an amphibian system after a severe renal insult.

While only a subset of the nephrectomized population was capable of successfully regenerating a fully coiled, functional pronephric organ, other individuals possessed smaller, uncoiled tubules that were also capable of reabsorbing BSA-AF488. It is likely that these shorter tubules had restored integration with the nephric coelom and so were able to receive filtered albumin. Presumably these tubules would continue to elongate if allowed to repair for more than three weeks. These observations indicate that a single population of nephrectomized tadpoles contains individuals in many stages of tubule restoration at any given time after injury, and further suggests that there is some stochasticity in the regenerative process employed by amphibian larvae after nephrectomy.

#### *An early apoptotic event occurs during proximal tubule regeneration*

We assessed levels of apoptosis at the site of injury by examining the expression of active Caspase 3 protein. As expected for any developing organism, low levels of apoptosis were detected in various tissues of all tadpoles examined, most notably in the gut and brain. In the renal region, 0-3 apoptotic cells could be found in unoperated control kidneys and contralateral kidneys in most nephrectomized tadpoles. For this reason, an increase in active Caspase 3 at the site of nephrectomy was determined by



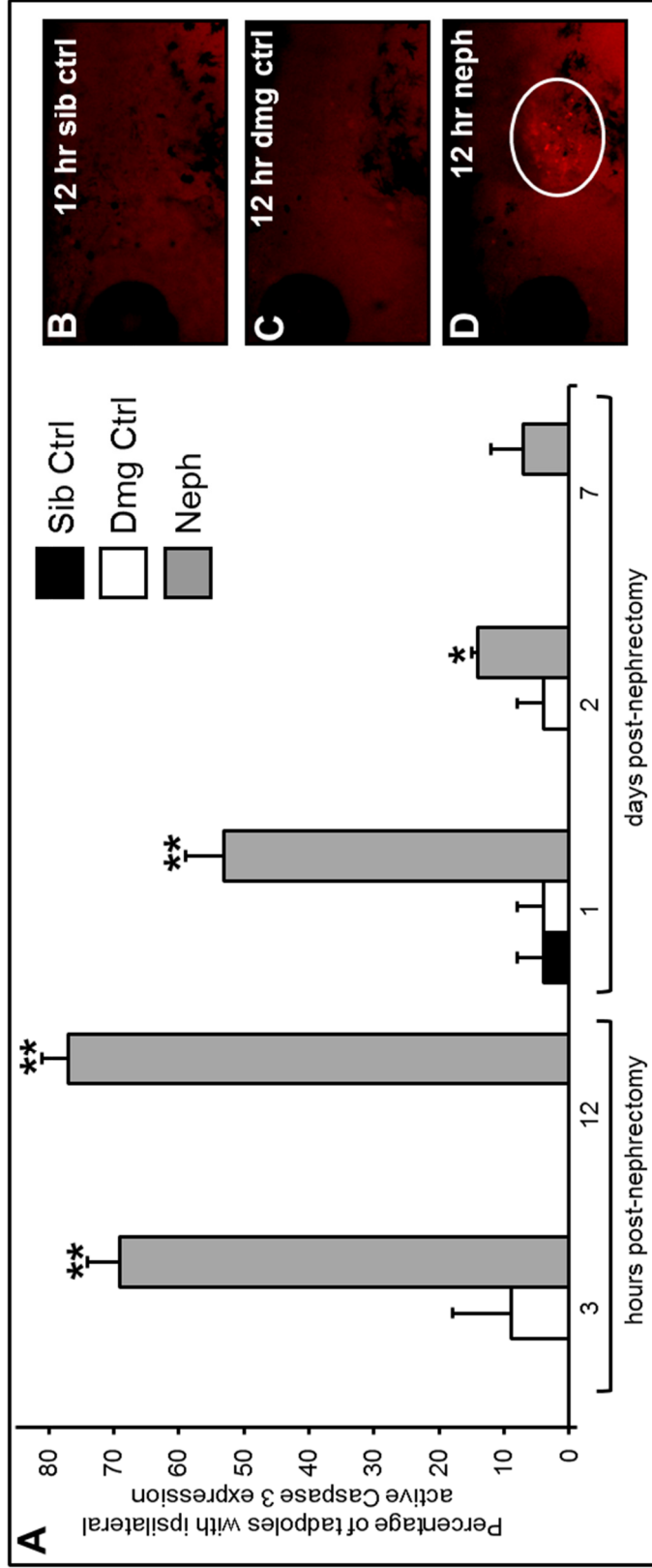
observing more active Caspase 3-positive cells on the ipsilateral side relative to the contralateral side. Damage control tadpoles and unoperated tadpoles were scored using the same criterion.

A dramatic increase of active Caspase 3-expressing cells was detected in the operated renal area in only the nephrectomized tadpole treatment group (Figure 2.4).

Apoptotic cells were observed on the ipsilateral side of nephrectomized tadpoles at 3 hours post-nephrectomy (hpn) and remained detectable in the majority of this population through the first 24 hours following tubule excision (Figure 2.4A). The maximum number of active Caspase-3 positive cells was observed at 12 hpn (Figures 2.4B-2.4D). Levels remained significantly elevated in nephrectomized tadpoles at 2 dpn, but returned to basal levels by 7 dpn. Strikingly, almost no active Caspase 3 protein was found in damage control tadpoles at any time-point examined (Figure 2.4A), suggesting that injury alone is not sufficient to induce apoptosis in this system. Apoptosis was also undetectable in unoperated control tadpoles. Thus, this early apoptotic event is part of a specific response to pronephric tubule excision.

#### *Xmmp-9 is expressed in a biphasic pattern after unilateral nephrectomy*

In addition to apoptosis, we also examined expression of the extracellular matrix (ECM) remodeler, matrix metalloproteinase 9 (*Xmmp-9*) during the first week after nephrectomy. As reported previously, small numbers of *Xmmp-9*-expressing cells are routinely observed in numerous tissues throughout development including: the epidermis, intestine, cloaca, tail fin and facial structures (Carinato et al., 2000). However, this

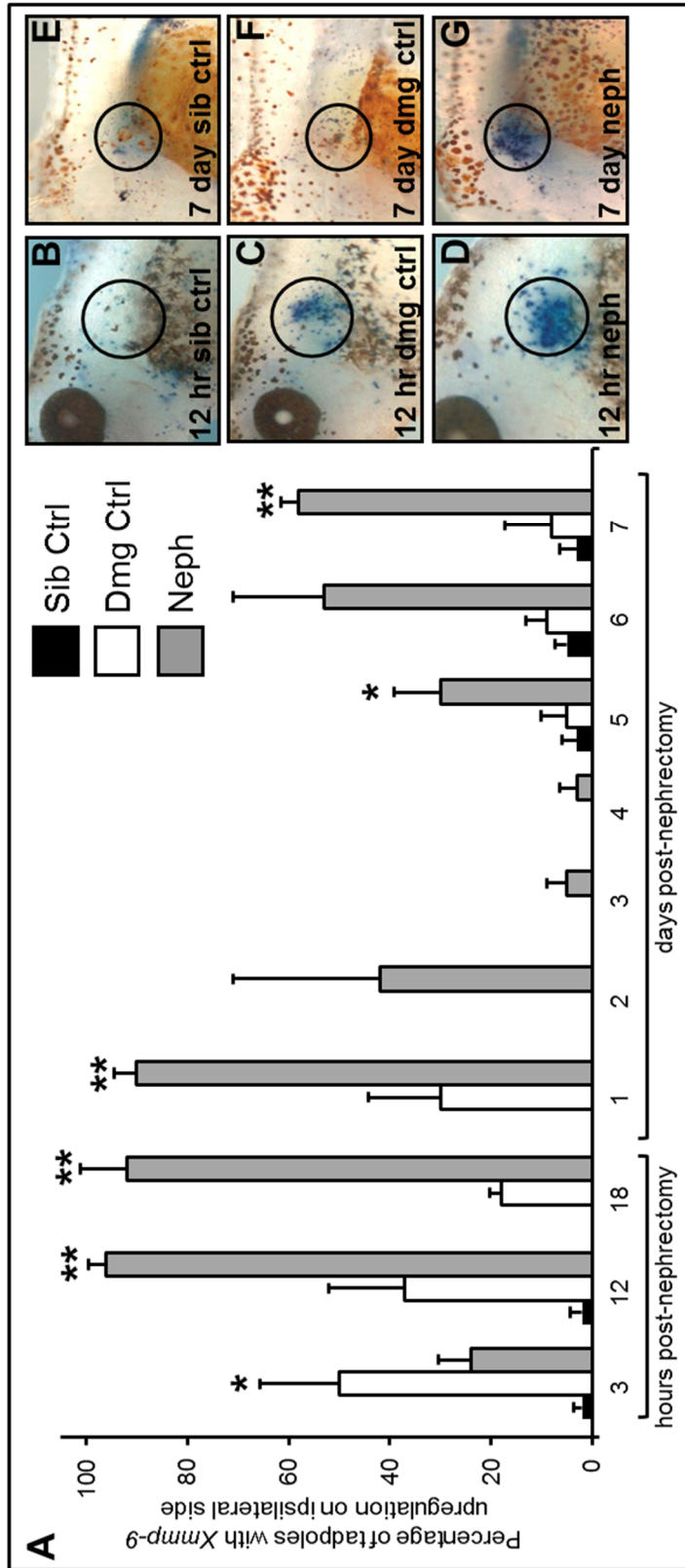


**Figure 2.4 Nephrectomized tadpoles have increased levels of apoptosis during the first 24 hours post-surgery.** A significant increase in the number of nephrectomized tadpoles expressing active Caspase 3 protein (as compared to both damage controls and unoperated sibling controls) was observed throughout the first day post-surgery (A). This number decreased significantly by the second day, and remained at low levels throughout the remainder of the first week post-surgery. 25-70 animals from each treatment group were assayed at each time-point examined. Error bars indicate standard error among 2-3 replicates/group. Asterisks denote single-factor ANOVA tests among different groups at the same time-point: \*,  $p < 0.05$ ; \*\*,  $p < 0.001$ . Photographs are lateral views (anterior to the left) of an unoperated sibling control (B), damage control (C), and nephrectomized tadpole (D), at 12 hours post-surgery. White circle indicates renal area with active Caspase 3-expressing cells.

baseline *Xmmp-9* expression was punctate, and was not concentrated in any one location. In contrast, upregulated *Xmmp-9* expression was observed in the ipsilateral renal area of nephrectomized tadpoles in a biphasic pattern.

The first phase of increased *Xmmp-9* expression began at 3 hpn and was sustained for the first 24 hours, with the vast majority of tadpoles displaying this phenotype between 12 hpn and 24 hpn (Figure 2.5A). Notably, the number of *Xmmp-9* positive cells in nephrectomized tadpoles also increased dramatically by 12 hpn (Figure 2.5D) and remained elevated through 24 hpn. As predicted by the role of *Xmmp-9* in wound healing (Carinato et al., 2000), damage control tadpoles also experienced upregulation of transcript levels on the ipsilateral side during the first 24 hours. However between 12 and 24 hours post-injury, significantly fewer damage control tadpoles than nephrectomized tadpoles had an increase in *Xmmp-9* at the site of renal injury (Figure 2.5A). Additionally, the level of transcript expression was also noticeably less in damage control tadpoles (compare Figures 2.5C and 2.5D). Unoperated sibling control tadpoles had no *Xmmp-9* upregulation during this first 24 hour phase (Figures 2.5A-2.5B). Ipsilateral *Xmmp-9* expression in all groups declined by 2 days post-nephrectomy, and remained low for the following 48 hours.

A second phase of ipsilateral transcript upregulation began at 5 dpn, but only in nephrectomized tadpoles. Both the proportion of the population re-expressing *Xmmp-9*, and the number of *Xmmp-9*-positive cells in each individual, increased over the next 48 hours, peaking at 7 dpn (Figure 2.5A and 2.5G). Strikingly, transcript upregulation was not detected on the ipsilateral side of damage control tadpoles during this phase (Figure



**Figure 2.5 *Xenopus laevis* tadpoles express *Xmmp-9* in a biphasic pattern after unilateral nephrectomy.** *In situ* hybridization analysis demonstrated that *Xmmp-9* expression was dramatically upregulated in both nephrectomized and damage control groups within 3 hours of injury. Significantly more nephrectomized tadpoles displayed this phenotype between 12 and 24 hours post-surgery. This number decreased by 2 days post-nephrectomy (dpn), and remained low until 5 dpn, at which time a second phase of *Xmmp-9* upregulation began on the ipsilateral side of only nephrectomized tadpoles. This phase persisted through 7 dpn (A). 25-70 animals from each treatment group were assayed at time-points examined. Error bars indicate standard error among 3-5 replicates/group. Asterisks denote single-factor ANOVA tests among different groups at the same time-point: \*,  $p=0.05$ ; \*\*,  $p<0.005$ . Photographs are lateral views (anterior to the left) of unoperated sibling controls (B and E), damage controls (C and F) and nephrectomized tadpoles (D and G), at 12 hours (B-D) or 7 days (E-G) post-surgery. Black circles indicate renal area in each tadpole.

2.5A and 2.5F). Unoperated sibling control kidneys also displayed no increase in transcript levels (Figure 2.5A and 2.5E).

## DISCUSSION

All nephric systems examined to date have been found to possess some form of reparative mechanism to offset the effects of acute damage. Regeneration of severely damaged or lost nephrons is less commonly observed, and as such is poorly understood. We are interested in understanding the renal repair response after this type of dramatic tissue loss. To accomplish this, we first needed to establish an injury model that would reproducibly create damage of this severity. In this study we describe a novel unilateral nephrectomy technique during which proximal tubules are preferentially excised from *X. laevis* pronephroi. We have demonstrated via morphological and molecular data that tadpoles have the capacity to regenerate functional proximal tubules, thus providing the first evidence of renal regeneration in an amphibian system. We also show that tubule restoration occurs via a process involving an early apoptotic event and the biphasic expression of the matrix metalloproteinase, *Xmmp-9*.

Using protein and transcript expression in various cell types of both renal and non-renal organs, we confirmed that proximal tubules were successfully targeted during surgery, while non-target tissues remained largely unaffected in nephrectomized tadpoles (Figure 2.1). 3G8 protein expression analyzed in 19 cohorts of nephrectomized tadpoles indicated that proximal tubule cells were either completely or partially removed in the majority of the population (Figure 2.1H). Conversely, normal proximal tubule 3G8

expression was observed in almost all damage control tadpoles (Figures 2.1C and 2.1H). In addition, although a few of the damage control sibling animals contained atypical, “partially ablated” 3G8 expression that resembled tubule structures, at 1 dpn no discernible tubules were observed in nephrectomized tadpoles that contained “partial 3G8 expression” (Figure 2.1A). These results clearly demonstrate that this nephrectomy technique is effective at specifically creating severe loss of pronephric proximal tubules.

It is important to note, however, that a small percentage (4%) of nephrectomized tadpoles examined at 1 dpn, appear to have normal 3G8 expression (Figure 2.1H). It is likely that the damage experienced by these few individuals is more subtle and thus not detectable by the macro-examination of protein expression. However, since the 3G8 pattern in these individuals is indistinguishable from that of unoperated control kidneys (Figure 2.1D), we must consider the possibility that these individuals will retain relatively normal morphology and physiology in both pronephroi throughout the three week repair period. Nonetheless, a significantly larger percentage of nephrectomized tadpoles was able to undergo complete tubule restoration by 21 dpn (Figure 2.2A), and these regenerates re-express genes associated with terminally differentiated kidneys (Figure 2.2C). 3G8 is specifically expressed in the apical membrane of proximal tubule cells (Vize et al., 1995), and so re-expression of this protein suggests that restored tubules regained not only morphological structure, but also cellular polarity.

Additionally, the ability of regenerates to reuptake albumin (BSA-AF488; Figure 2.3E) indicates that these tissues are comprised of lumenized cells. Albumin reuptake from the glomal/glomerular filtrate occurs via an active process involving multi-ligand endocytic receptors specifically localized to the apical plasma membrane of proximal

tubules segments of both pronephroi (Christensen et al., 2008; Zhou and Vize, 2004), as well as mesonephric and metanephric kidneys (Lazzara and Deen, 2007; Verroust et al., 2002; Birn et al., 2000; Lauriola et al., 1986). The reuptake of BSA-AF488 by restored proximal tubules indicates that these regenerates have regained luminal flow of glomerular filtrate and are re-expressing crucial endocytic proteins.

Interestingly, the proportion of 21 dpn tadpoles that had no detectable 3G8-positive tissue on the operated side is comparable to the percentage of the 1 dpn population lacking 3G8-positive tubules (Figure 2.2A). This correlation suggests that tissue renewal may rely on the presence of surviving proximal tubule cells. Similar conclusions have been previously made after examining renal responses to both nephrotoxic injury (Kays and Schnellmann, 1995; Wallin et al., 1992; Kovacs et al., 1982; Cuppage et al., 1972) and ischemia/reperfusion (Humphreys et al., 2008; Witzgall et al., 1994; Venkatachalam et al., 1978) in metanephric nephrons (Humphreys et al., 2011; Bonventre, 2003; Nony and Schnellmann, 2003). Surviving proximal tubule cells have also been shown to be important in repair events during human acute tubular necrosis (Nadasdy et al., 1995). It is likely that pronephric proximal tubule repair is similar to these metanephric systems, and thus also requires the survival of some proximal cells in order for tubule regeneration to occur. We postulate that the tubule regeneration observed in this study occurred in individuals that experienced partial nephrectomy of proximal tubules (Figure 2.2A, grey bar).

While metanephric kidneys are able to repopulate denuded basement membranes that remain intact after injury, these kidneys are unable to recover after dramatic loss of renal mass. This study, however, provides the first evidence of pronephric kidneys

regenerating functional tubule architecture after dramatic loss of renal tissue. It is likely that the less complex nature of the pronephric kidney makes it amenable to this extensive restoration, thereby making it a suitable organ to investigate kidney repair. Moreover, since severe kidney damage in humans is often accompanied by an intense fibroproliferative response (Liu, 2011), which is absent in nephrectomized tadpoles, elucidating the mechanisms that contribute to this pronephric regeneration will have important implications in innovating alternative treatments for AKI and CKD. For this reason, we examined important repair events that occur upon nephrectomy in this pronephric system.

Apoptosis or programmed cell death (PCD) is a non-necrotic mode of cellular disposal that is critical in amphibian development and is especially prevalent during metamorphosis (Nakajima et al., 2005). Notably, apoptosis has also been observed during many amphibian regenerative processes (Tseng et al., 2007; Yoshii et al., 2007; Gargioli and Slack, 2004; Bettencourt-Dias et al., 2003; Flink, 2002; Carlson et al., 2001; Gardiner et al., 1999; Lo et al., 1993). We have also detected an increase in apoptosis that is restricted to the first 24 hours after surgery in the majority of nephrectomized tadpoles (Figure 2.4). In contrast, there was no significant increase in apoptosis in damage control tadpoles indicating that PCD is not a general response to injury. Interestingly, apoptosis was also observed in tubule epithelia during the first 24 hours after induced regeneration of non-infarcted atrophic rat kidneys (Gobé et al., 1995). It is likely that this activity is a critical precursor to kidney regeneration as has been found in other systems. For example, Tseng et al. (2007) demonstrated that tail regeneration in *X.*



*laevis* tadpoles cannot proceed if apoptosis is inhibited during the first 24 hours post-tail amputation.

The timing of apoptosis after nephrectomy overlaps with the wound healing process, which may indicate a role for PCD in creating a permissive environment prior to regeneration. Although this permissive environment model is well-established in other regenerative systems (Zukor et al., 2011; Tucker et al., 2008; Price and Allen, 2004), the underlying role of apoptosis during early regenerative events is still unclear. It is possible that cell death is critical for eliminating damaged tissue prior to stimulation of renal restoration, or as suggested by Tseng and colleagues (2007), that apoptosis serves to destroy a subpopulation of cells that may normally inhibit regeneration. A third possibility is that PCD may be critical in limiting inflammatory damage, with a lack of apoptosis resulting in scar formation and perpetuation of renal disease (Gobe and Buttyan, 2002). This possibility is especially attractive considering that regenerative success is often thought to be the alternative to scarring (Price and Allen, 2004; Harty et al., 2003; Poss et al., 2002).

In mammalian metanephric kidneys, which have no neonephrogenic properties, injury typically results in extensive inflammatory activity leading to extensive renal fibrosis (Liu, 2011; Hewitt et al., 2008; Wynn, 2008). However, the immune system found in mammals is more highly evolved than that of anuran larvae, as well as adult urodeles (Robert and Cohen, 1998), both of which are well-established model systems for regeneration. Since the observed apoptotic event coincides with the onset of inflammation after injury, it is likely that apoptosis may modulate the inflammatory response to allow for tissue regeneration instead of fibrosis.

Establishing a fibrosis-free permissive environment for regeneration also involves remodeling of the ECM to facilitate cellular migration and cell-cell signaling, as well as to provide a scaffold for tissue reconstruction. Many of these ECM modifications are performed by the MMPs, a multi-member family of zinc-dependent endopeptidases (Johnson et al., 1998; Matrisian, 1992). MMP-9 in particular has been implicated in regeneration, with levels upregulated both at the onset of wound healing (Liu et al., 2009; Carinato et al., 2000; Yang et al., 1999; Fini et al., 1998; Salo et al., 1994) and after the completion of wound healing (Yang et al., 1999). We speculate that the first 12 hours of *Xmmp-9* expression observed after injury in both nephrectomized and damage control tadpoles mediates wound healing, which also coincides with the length of time for skin wound closure after injury. Strikingly, from 12-24 hours *Xmmp-9* continues to be robustly expressed in nephrectomized tadpoles alone (Figure 2.5A), suggesting that MMP-9 upregulation during this period may be a specific response to loss of tubule tissue. Previous work demonstrated that suppressed MMP-9 activity in damaged metanephric kidneys results in excessive collagen deposition (Bengatta et al., 2009; Chromek et al., 2004; Chromek et al., 2003; González-Avila et al., 1998). Consistent with our findings, it is possible that MMP-9 plays a similar role in the pronephros and serves to minimize fibroproliferation, thereby facilitating the creation of the proper environment for regeneration to occur. This is even more likely given the ability of MMP-9 to cleave interstitial Type-I collagen (Bigg et al., 2007), a predominant protein found in renal scar tissue (Huang et al., 2011).

The second phase of *Xmmp-9* expression (5-7 dpn), which was also only observed in nephrectomized tadpoles (Figure 2.5A), indicates that this upregulation is involved in

tubule restoration, and is not a general response to damage. A similar biphasic pattern of *Mmp-9* was observed in axolotl limb regeneration after wound healing was completed, with *Mmp-9* facilitating the creation of new cartilage (Yang et al., 1999). In the renal system, MMP-9 may be playing a similar role in the reconstruction of tubule tissue. This is an especially appealing possibility given that MMP-9 is critical for tubulogenesis and branching during development of the kidney (Arnould et al., 2009; Lelongt et al., 1997). Over a decade ago, researchers began to examine molecular profiles of renal repair, and detected similarities in gene expression during mammalian metanephric development and epithelial renewal in injured adult kidneys (Reimschuessel, 2001). Since the developmental program of kidney organogenesis is well conserved among vertebrates (Dressler, 2006; Kuure et al., 2000; Drummond et al., 1998; Vize et al., 1997), then this may also be true of the regenerative process.

Interestingly, murine MMP-9 has been shown to stimulate branching morphogenesis during kidney development by protecting cells from apoptosis (Arnould et al., 2009). Similarly, previous work on acute kidney injury in mice suggests that MMP-9 is critical to reduce the incidence of apoptosis during later stages of renal repair (Bengatta et al., 2009). Since the upregulation of both MMP-9 and apoptosis overlap in this injury model, it is possible that they are intricately linked in a similar manner during this restorative process. Further investigation into this possibility is necessary in order to elucidate the roles that MMP-9 and apoptosis play in this pronephric kidney regeneration process following nephrectomy. Ultimately, this line of inquiry has important implications for clinical research aimed at attenuating the effects of renal disease in humans.

## **ACKNOWLEDGEMENTS**

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## CHAPTER THREE

### **Examining the roles of apoptosis and XMMP-9 activity in the early stages of pronephric regeneration in *Xenopus laevis***

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While the initial studies in Chapter 2 described the expression of the *matrix metalloproteinase 9* transcript and the occurrence of cell death during the first week after nephrectomy, it did not characterize the roles of these events in renal regeneration. Thus, we examined the function of both XMMP-9 activity and apoptosis on this repair process, with particular focus on the first phase of *Xmmp-9* expression (0-1 dpn), which also coincides with the peak in detectable apoptosis. In this chapter, we demonstrate that while an early apoptotic event may be detrimental to the repair process, XMMP-9 activity promotes tubule regeneration during this early window of repair.

## **ABSTRACT**

Renal proximal tubules are particularly vulnerable to chemotoxic, hypoxic and obstructive damage, which often lead to acute renal injury or chronic kidney disease. However, under permissive conditions, proximal tubules have been found to re-epithelialize denuded basement membranes in mammalian kidneys and regenerate in adult fish. Recently, we demonstrated that pronephric kidneys in *Xenopus laevis* tadpoles are also able to regenerate proximal tubules after partial unilateral nephrectomy. Elucidating the cellular mechanisms that regulate these reparative and regenerative events would be beneficial for the development of renal disease therapies, and would also contribute to distinguishing between species-specific adaptations in kidney repair mechanisms. In this study we examine the roles of Matrix metalloproteinase-9 activity and apoptotic cell death in the early stages of tubule regeneration (1 day post-nephrectomy). Via loss-of-function assays, we show that an early increase in apoptosis

may suppress proximal tubule repair, while early XMMP-9 activity is required for successful regeneration.

## **INTRODUCTION**

Renal tubule cells possess a remarkable capacity for repair after diverse types of damage including: chemotoxic (Diep et al., 2011; Salice et al., 2001; Augusto et al., 1996; Reimschuessel and Williams, 1995; Nonclercq et al., 1992; Laurent et al., 1988; Boti et al., 1982; Cuppage et al., 1972; Cuppage and Tate, 1967), mechanical (Caine and McLaughlin, 2013; Elger et al., 2003), ischemia/reperfusion (Devarajan, 2005; Padanilam, 2003; Sheridan and Bonventre, 2001; Witzgall et al., 1994), unilateral ureteral obstruction (UUO) (Wang et al., 2010; Cochrane et al., 2005; Yang et al., 2002), and growth factor deficiency (Bonegio and Lieberthal, 2002). In amniotes, renal repair is limited to re-epithelialization of injured tubules, which is often accompanied by some degree of fibrosis (Hewitt et al., 2008; Chromek et al., 2003). In fish and amphibians, however, tubule regeneration is more extensive and typically occurs in the absence of fibroproliferation (Caine and McLaughlin, 2013; Diep et al., 2011; Elger et al., 2003; Salice et al., 2001).

The regenerative capacity of tissues is largely defined by cell signaling during the initial inflammatory phase of repair. This determines the extent of fibrosis that occurs during the subsequent proliferative phase (Harty et al., 2003), a process primarily governed by extracellular matrix (ECM) remodeling at the site of injury (Liu, 2011; Wynn, 2008; Raghow, 1994). ECM remodeling is predominantly regulated by the multi-

member family of zinc-dependent endopeptidases called matrix metalloproteinases (MMPs) (Johnson et al., 1998; Matrisian, 1992), which includes interstitial collagenases, stromelysins, matrilysins, elastases, gelatinases, secreted MMPs and membrane-type MMPs. Together these proteases synergistically degrade nearly all ECM components and thus play a role in several developmental and pathophysiological processes including embryogenesis, morphogenesis, angiogenesis, tumor cell invasion, inflammation, wound healing, and fibrosis (Harrison et al., 2004; Lelongt et al., 2001b; Andrews et al., 2000; Tanney et al., 1998).

Among the 28 MMPs identified to date (Wang et al., 2010), the gelatinase MMP-9 has repeatedly been found to mediate fibrosis in several diseased mammalian organs including the heart (Matsumoto et al., 2009), liver (Prakobwong et al., 2010), lungs (Atkinson and Senior, 2003), and metanephric kidneys (Zeisberg and Neilson, 2010). Recently, we have shown that *Xmmp-9* is also expressed in response to damage in embryonic (pronephric) kidneys in *Xenopus laevis* (Caine and McLaughlin, 2013).

While the structural and biochemical aspects of the MMP-9 enzyme are well-characterized (Van Den Steen et al., 2002), its role in renal repair remains unclear. This is due in large part to the inconsistent anti-fibrotic effects observed in the interstitium of metanephric kidneys. While some studies have demonstrated a protective role for MMP-9 in renal disease (Bengatta et al., 2009; Liu et al., 2009; Chromek et al., 2003; Lelongt et al., 2001a; González-Avila et al., 1998), others have either seen no effect due to MMP-9 deficiency (Andrews et al., 2000), or have described a detrimental role in which MMP-9-mediated ECM degradation pathologically disrupts the tubular basement membrane (TBM) (Kunugi et al., 2011; Wang et al., 2010; Yao et al., 2009; Yang et al., 2002;



McMillan et al., 1996). Pleiotropic effects of MMP-9 (both protective and detrimental) may also depend on the stage of repair (Chromek et al., 2003; Lelongt et al., 2001b; Yang et al., 1999) and the model of disease (Lelongt et al., 2001b).

Interestingly, in both kidney repair (Bengatta et al., 2009) and development (Arnould et al., 2009), MMP-9 has been found to inhibit apoptosis, the caspase-mediated form of programmed cell death (PCD) (Mannello et al., 2005). Apoptosis occurs during development of many animal tissues among several phyla, where it functions to eliminate cells targeted for degradation (Baehrecke, 2002; Jacobson et al., 1997). This process is dissimilar from necrosis in that it is highly regulated and progresses without eliciting a potentially harmful inflammatory response (Elmore, 2007). For this reason, apoptosis is often associated with cellular death that confers an advantage to an organism such as the differentiation of digits in the developing limb (Zuzarte-Luis and Hurle, 2005) or the remodeling of various organs during amphibian development (Hensey and Gautier, 1998) and metamorphosis (Nakajima et al., 2005). PCD is also an important feature of kidney development (Carev et al., 2006; Hayashi and Araki, 2002; Pole et al., 2002; Araki et al., 1999), and is responsible for removing dead cells in the nephrogenic zone where new nephrons are produced (Coles et al., 1993).

After renal injury, cell death is typically an immediate cellular response (within the first 1-2 days of damage), however this event has historically been described as necrosis (Donohoe et al., 1978; Venkatachalam et al., 1978; Cuppage et al., 1972; Cuppage and Tate, 1967). Following the definitive publication that promoted the term “apoptosis” (Kerr et al., 1972), the distinction between these two modes of cell death in renal repair has become clearer. While both events can occur in response to renal

damage (Lieberthal et al., 1998), apoptosis appears to be the predominant mode of PCD induced in injured renal structures (Gobe and Buttyan, 2002).

Interestingly, the role of apoptotic cell death varies in several models of kidney disease. Demonstrating a protective role in renal repair, apoptosis has been found to minimize the deleterious effects of inflammation by reducing the number of granulocytes at the site of injury (Chromek et al., 2004; Gobe and Buttyan, 2002; Savill, 1997) and may also contribute to the remodeling of injured tubules during renal repair (Lieberthal et al., 1998). Conversely, the detrimental effects of apoptosis have also been described in pathogenesis of mammalian renal disease (Gobe and Buttyan, 2002), where it contributes to TBM destruction and fibrosis (Kunugi et al., 2011; Wang et al., 2010; Yang et al., 2002).

Apoptosis has also been observed after unilateral pronephrectomy in *X. laevis* (Caine and McLaughlin, 2013), however it is still unknown what role this event may be playing in the regeneration observed in this system. Since *Xmmp-9* is also observed during the first 24 hours after pronephric damage (Caine and McLaughlin, 2013), it is possible that this protease may be involved in regulating apoptosis in this renal system as seen in the mammalian kidney (Bengatta et al., 2009). To investigate this possibility, we used pharmacological inhibitors of both apoptosis and XMMP-9 activity to examine the loss-of-function effects on pronephric proximal tubule regeneration. Our results suggest that the apoptotic event observed during the first 24 hours post-nephrectomy, proceeds through an extrinsic PCD pathway, and that inhibition of this event correlates to greater regenerative success of renal tubules. We also demonstrate that MMP-9 activity within 24 hours of renal damage promotes renal tissue regeneration.

## EXPERIMENTAL PROCEDURES

### *Xenopus laevis* Tadpoles

All experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals and were approved via the Institutional Animal Care and Use Committee (IACUC) at Tufts University. *Xenopus laevis* tadpoles were obtained via a standard *in vitro* fertilization protocol (Caine and McLaughlin, 2013), staged according to Nieuwkoop and Faber (NF, 1994), and reared in 0.1X Marc's Modified Ringer's solution (MMR; 10 mM NaCl, 0.2 mM KCl, 0.1 mM CaCl<sub>2</sub>, 0.2 mM MgCl<sub>2</sub>, 0.5 mM HEPES, 1  $\mu$ M EDTA, pH 7.4) (Kay and Peng, 1991).

### *Tail Amputation*

NF stage 37/38 tadpoles were anaesthetized with 0.04% tricaine in 0.1X MMR and tails were amputated with a scalpel with the aid of a dissecting microscope. To maximize consistency among amputated tadpoles, tails were cut posterior to the proctodeum, where the tail muscle begins to taper, as previously described (Tseng et al., 2007). Tadpoles were then transferred to media containing either inhibitor or vehicle (see below) or 0.1X MMR and reared at room temperature for 9 days to maximize time allowed for tail regrowth. Larvae were not fed during these experiments. At the end of the experiment, both amputated and unamputated control tadpoles were euthanized with tricaine, fixed for one hour at room temperature in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO<sub>4</sub>, 3.7 % formaldehyde), rinsed in 1X phosphate buffered saline (PBS), and gradually dehydrated to 100% methanol for storage at -20°C.

### *Partial Unilateral Pronephrectomy*

NF stage 37/38 tadpoles were anaesthetized with 0.04% tricaine in 0.1X MMR, and pronephric proximal tubules were excised as previously described (Caine and McLaughlin, 2013). Stage-matched unoperated sibling control tadpoles were also collected for each assay. NF stage 48 and older tadpoles were fed daily with 0.008% Sera Micron powdered growth food in 0.1X MMR.

### *Inhibitor Exposures*

Loss-of-function experiments were performed via exposure to either an apoptotic inhibitor or an inhibitor of MMP-9 activity. Inhibitor stocks were prepared in DMSO and all exposures were conducted at the indicated concentrations by dissolution in 0.1X MMR. Apoptosis was inhibited with 20 $\mu$ M 2,2'-Methylenebis-1,3-cyclohexanedione (M50054; Calbiochem) in 0.05% DMSO. XMmp-9 activity was inhibited with either 30 $\mu$ M GM6001 (Calbiochem) in 0.012% DMSO, or 10 $\mu$ M SB-3CT (MMP-2/MMP-9 Inhibitor IV; Calbiochem or Santa Cruz Biotechnology) in 0.06% DMSO. Exposures were performed for 24 hours post-tail amputation or post-nephrectomy. Individual tadpoles were transferred to exposure media within 5 minutes of tail amputation or within 2 hours of tubule excision. Exposures were terminated by transferring larvae to new dishes and rinsing three times with fresh 0.1X MMR. To assess the effects of inhibitors on tadpole development, behavior and/or mortality, exposures were also performed on unamputated or unnephrectomized sibling controls.

### *Detection of Apoptosis*

Levels of apoptosis were assessed by expression of active Caspase-3 protein on the ipsilateral/left side of nephrectomized/unoperated sibling control tadpoles. At 1 day post-surgery, tadpoles were euthanized with tricaine, fixed in MEMFA (0.1M MOPS, pH 7.4, 2mM EGTA, 1mM MgSO<sub>4</sub>, 3.7% formaldehyde) for one hour at room temperature, rinsed in 1X phosphate buffered saline (PBS) and gradually dehydrated to 100% methanol until needed. Active Caspase-3-positive cells were detected via whole-mount immunohistochemistry as described previously (Caine and McLaughlin, 2013) using anti-activated Caspase-3 primary antibody (Abcam #ab13847 or BD Biosciences Pharminogen #559565) and Alexa Fluor 555-conjugated goat anti-rabbit IgG secondary antibodies (1:300; Invitrogen). All tadpoles were examined under fluorescent microscopy, transferred to 1X PBS and stored at 4°C. To analyze apoptosis, the number of active Caspase-3-positive cells in the nephric region was counted on both sides. Since baseline levels of 1-3 active Caspase-3-positive cells can be found in unmanipulated kidneys (Caine and McLaughlin, 2013), in this study we define an increase in apoptotic response as the presence of 4 or more fluorescent cells in the renal area.

### *Assessment of Proximal Tubule Regeneration*

Regenerative success was determined by calculating the percentage of tadpoles from each treatment (unexposed, vehicle-control, inhibitor-exposed) with coiled, 3G8-positive proximal tubules (normal tubule phenotype) at 21 dpn. 3G8 protein expression was detected by whole-mount immunohistochemistry as previously described (Caine and McLaughlin, 2013) using anti-3G8 primary antibody (generously provided by Elizabeth

Jones, Ph.D.), and alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibodies, which was developed with 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium (1:600 BCIP/1:600 NBT; Roche Diagnostics). At 21 dpn, it is difficult to distinguish between true regenerated kidneys and inadequately operated kidneys that have retained normal tubule phenotype for the extent of the assay. Thus, to account for the proportion of each cohort that may have been insufficiently nephrectomized, representative tadpoles were also collected at 1 dpn and assayed for 3G8 expression. The percentage of tadpoles at 21 dpn that successfully regenerated proximal tubules was calculated for each group/replicate by first subtracting for the percent of 1 dpn tadpoles that retained full 3G8 expression.

#### *Detection of *Xmmp-9* after Injury*

To examine the expression of *Xmmp-9* after injury, tadpoles were randomly assigned to either the unmanipulated sibling control group, or one of 5 injury groups: nephrectomy, tail amputation, skin incision along the lateral flank, somite puncture, or gut puncture. Representative individuals were then euthanized with tricaine at specific time-points and fixed in MEMFA for one hour at room temperature. To localize *Xmmp-9* transcripts (Carinato et al., 2000), whole mount *in situ* hybridization was performed on tadpoles (NF stages 37-40/41) via hybridization with digoxigenin-labeled antisense riboprobes as described previously (Harland, 1991). mRNA expression was detected via an anti-digoxigenin antibody conjugated to AP (1:1500; Roche Diagnostics) and a blue BCIP/NBT precipitate. Tadpoles were post-fixed overnight in MEMFA at 4°C, gradually dehydrated to methanol and stored at -20°C.

### *Examination of MMP-9 Protein*

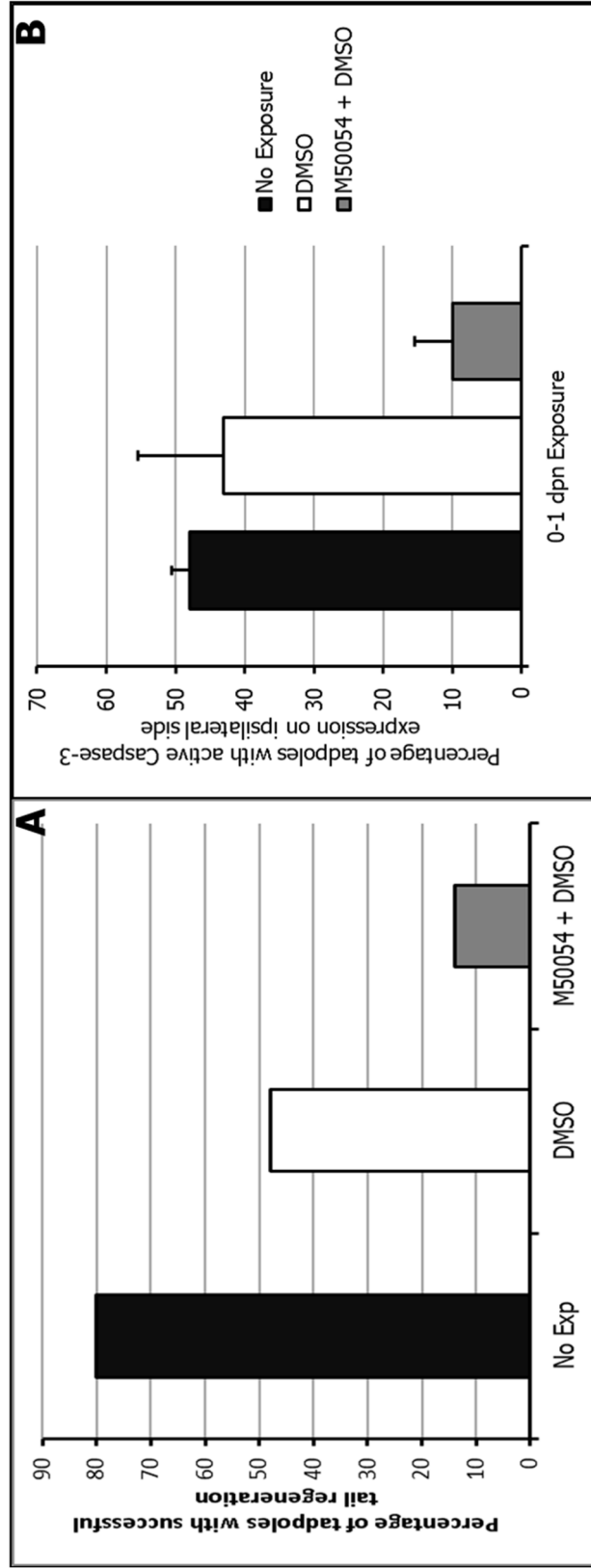
To examine localization of MMP-9 protein, whole mount immunohistochemistry was performed on 1 dpn tadpoles (NF stage 40/41) as previously described (Caine and McLaughlin, 2013) using anti-MMP-9 primary antibody (Cell Signaling Technology) and Alexa Fluor 555-conjugated goat anti-rabbit IgG secondary antibodies (1:300; Invitrogen). All tadpoles were examined under fluorescent microscopy, transferred to 1X PBS and stored at 4°C. Representative individuals were photographed with a Nikon SMZ1500 stereomicroscope and Spot Insight Color digital camera and software.

## **RESULTS**

### *Inhibition of apoptosis during amphibian tissue regeneration*

To investigate the role of apoptosis in repair, we first confirmed the efficacy of the apoptotic inhibitor M50054, in our system. No unusual behavior or gross morphological effects were observed in any amputated or unamputated tadpoles exposed to either M50054 or the vehicle (DMSO). However, as previously reported (Tseng et al., 2007), tail regeneration was ablated in the majority of tadpoles exposed to this apoptotic inhibitor during the first 24 hours post-amputation (Figure 3.1A).

We also confirmed that M50054 would inhibit the elevated levels of apoptotic cells previously observed on the ipsilateral side of tadpoles at 1 day post-nephrectomy (dpn) (Caine and McLaughlin, 2013). Exposure to M50054 dramatically reduced the percentage of nephrectomized tadpoles with upregulated active Caspase 3, as compared



**Figure 3.1 Exposure to the apoptotic inhibitor M50054 blocks apoptosis in the tail and the wound site after nephrectomy in *Xenopus laevis* tadpoles.** Amputated tails of *Xenopus laevis* tadpole tails are unable to regenerate normally in the presence of 20 $\mu$ M M50054 (in 0.05% DMSO) (A). Exposure to M50054 from 0-1 day post-nephrectomy also blocks the expression of active Caspase 3 on the ipsilateral side of nephrectomized tadpoles at 1 dpn (B). Error bars indicate standard error between 2 replicates, n=39-40/group.



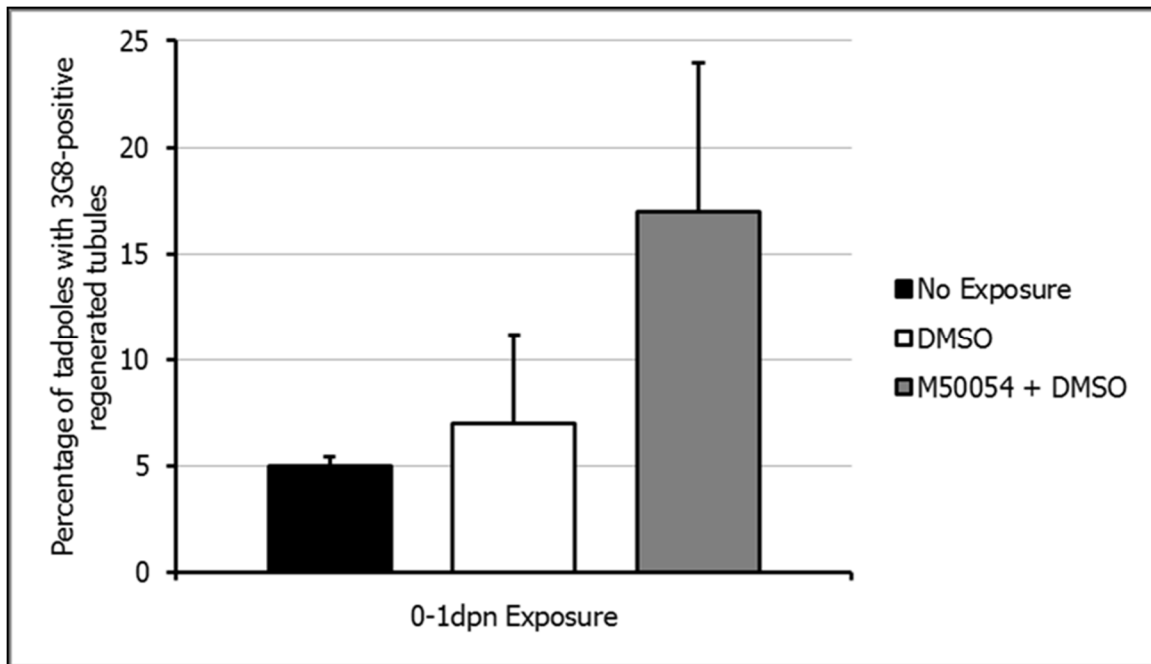
to both vehicle-exposed and unexposed sibling controls (Kruskal-Wallis test,  $p = 0.18$ ; Figure 3.1B).

*Early apoptosis may be detrimental for proximal tubule regeneration*

Since a subset of nephrectomized tadpoles are able to fully regenerate a pronephric kidney three weeks post-surgery (Caine and McLaughlin, 2013), we investigated whether apoptotic events observed between 0-1 dpn were essential for successful regeneration. At 21 dpn, all exposed and unexposed regenerated kidneys were phenotypically comparable to unoperated control kidneys, possessing fully coiled tubules and expressing the differentiated proximal tubule protein, 3G8 (data not shown). However, 0-1 dpn M50054-exposure substantially increased the percentage of nephrectomized tadpoles with regenerated kidneys, as compared to both vehicle-exposed and unexposed sibling controls (Figure 3.2). While the differences among these treatment groups is not statistically significant (Kruskal-Wallis test,  $p = 0.29$ ), the trend in these data suggest that an early apoptotic event after nephrectomy may hinder the success of subsequent tubule repair.

*Early XMMP-9 activity promotes proximal tubule regeneration*

In addition to active Caspase-3, *Xmmp-9* expression is also upregulated during the first 24 hours after nephrectomy (Caine and McLaughlin, 2013), thus we also examined the function of this protease during proximal tubule repair. No unusual behavior or gross morphological effects were observed in nephrectomized or unnephrectomized tadpoles exposed to either GM6001 or the vehicle DMSO. Some GM6001-, SB3CT- and 0.012%

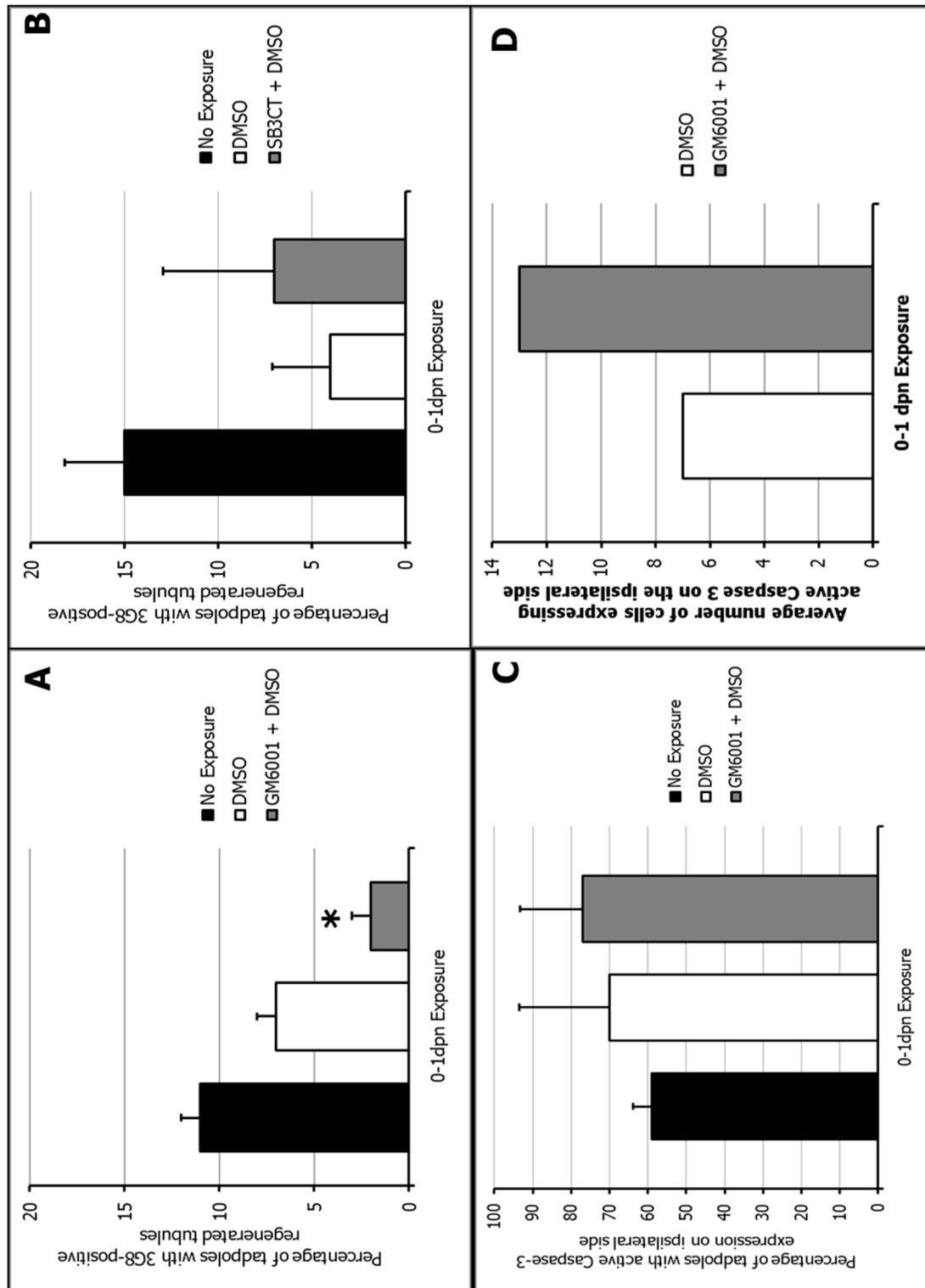


**Figure 3.2 Exposure to the apoptotic inhibitor, M50054, from 0-1 day post-nephrectomy, promotes tubule regeneration in *Xenopus laevis* tadpoles.** Tadpoles were allowed to recover for three weeks post-nephrectomy then assayed for the presence of fully coiled, regenerated tubules via expression of the differentiated proximal tubule protein 3G8 on the ipsilateral (operated) side. Exposures were performed with 20 $\mu$ M M50054 in 0.05% DMSO. Error bars indicate standard error among 3 replicates, n= 59-63/group.

DMSO-exposed nephrectomized tadpoles were found on 1 dpn with unhealed skin at the surgical site. However, this condition did not affect a significant number of exposed tadpoles in any group (SB-3CT Kruskal-Wallis test,  $p=0.09$ ; GM6001 Kruskal-Wallis test,  $p = 0.76$ ), and was resolved within 24-48 hours in all individuals.

Early (0-1 dpn) exposure to GM6001, a broad-spectrum hydroxamic MMP inhibitor (Galaray et al., 1994b), significantly inhibited the percentage of tadpoles with regenerated (fully coiled, 3G8-positive) proximal tubules in the nephrectomized population (Kruskal-Wallis test,  $p = 0.04$ ; Figure 3.3A). Tubule regeneration was also reduced after 0-1 dpn exposure to the compound SB-3CT, a mechanism-based inhibitor specific to the gelatinases (MMP-2 and MMP-9) (Brown et al., 2000), as compared to both vehicle-exposed and unexposed sibling controls (Figure 3.3B). Although the differences among the treatment groups in these preliminary SB-3CT exposures were not statistically significant (Kruskal-Wallis test,  $p = 0.50$ ), this variance may be attenuated by further iterations of these assays. Combined, these results are consistent with a role for XMMP-9 activity during the early phases of repair (0-1 dpn window) in promoting successful regeneration of pronephric proximal tubules.

One potential role of XMMP-9 activity during this period is the mediation of apoptosis in the wound microenvironment. To test this hypothesis, we examined the effect of GM6001 exposure on levels of ipsilateral apoptosis in 1 dpn tadpoles. While there was a slight increase in the percentage of GM6001-inhibited tadpoles with increased levels of ipsilateral active Caspase-3, this trend was not significantly different from the vehicle-exposed or unexposed treatment groups (Figure 3.3C; Kruskal-Wallis test,  $p = 0.77$ ). Additionally, there were no significant differences in the average number



**Figure 3.3. Legend on next page.**

**Figure 3.3 Inhibition of XMMP-9 activity dramatically reduces the ability of pronephric kidneys to regenerate in *Xenopus laevis* tadpoles.** Nephrectomized tadpoles were exposed to 30 $\mu$ M GM6001 (in 0.012% DMSO) (A) or 10 $\mu$ M SB-3CT (in 0.06% DMSO) (B) from 0-1 day post-nephrectomy (dpn) and then assayed at 21 dpn for the presence of fully coiled, regenerated tubules via expression of the differentiated proximal tubule protein 3G8 on the ipsilateral (operated) side. Asterisk in (A) denotes Kruskal-Wallis test among all three treatment groups,  $p = 0.04$ . Nephrectomized tadpoles were also exposed to GM6001 from 0-1 dpn then assayed for the presence of active Caspase 3 on the ipsilateral side at 1 dpn (C-D). GM6001 exposure had no effect on the percentage of tadpoles with upregulated active Caspase 3-expression (C) nor the number of active Caspase 3-positive cells expressed on the ipsilateral side of GM6001-exposed tadpoles (D). Error bars indicate standard error among the following: (A) N=4, n= 85-87 /treatment; (B) N=2, n= 39-52/treatment; (C) N=2, n=37-39.

of detectable active Caspase-3-positive cells among the treatments (2-tailed Student's *t*-test,  $p = 0.62$ ; Figure 3.3D).

## DISCUSSION

Proximal tubules are the most anterior nephron segments of all vertebrate kidneys (pronephros, mesonephros and metanephros), and perform critical reabsorption of water and solutes from the glomerular filtrate (Christensen et al., 2008; Zhou and Vize, 2004). Because of this function and their location in the nephron, these segments are the first to be exposed to nephrotoxic substances present in the filtrate that are destined for excretion. Oftentimes small but substantial levels of these noxious chemicals may be taken up by proximal tubule cells (Mingeot-Leclercq and Tulkens, 1999), leading to toxicity and subsequent sloughing of dead cells into the nephron lumen (Liu et al., 2002). In the lumen, cellular debris may accumulate into casts, forming obstructions that perpetuate tubule dysfunction (Humes et al., 1989). Additionally, cells of proximal tubule segments possess a lower capacity for glycolytic-generation of ATP than do distal segments, and therefore are more susceptible to hypoxia caused by ischemia (Lieberthal and Nigam, 1998; Venkatachalam et al., 1981; Venkatachalam et al., 1978). Perhaps as an adaptive response to these limitations, proximal tubule cells tend to be renewed more readily than other parts of the nephron. However, the mechanisms that trigger regeneration or re-epithelialization of proximal tubules are not well understood. To determine potential mechanisms that may be activated early in pronephric tubule repair,

this study focuses on examining the roles of cell death and matrix metalloproteinase activity during the first day post-nephrectomy.

Recently we showed a peak in cell death between 0 and 1 dpn that appeared to be a specific response to proximal tubule excision (as opposed to general tissue damage) (Caine and McLaughlin, 2013). This elevated level of apoptotic death is successfully blocked by M50054 exposure (Figure 3.1B), a potent inhibitor of apoptosis in *X. laevis* as demonstrated by its ability to inhibit tail regeneration (Figure 3.1A and Tseng et al., 2007). M50054 inhibits Caspase 3 activation involved in Fas ligand-induced cell death and in apoptosis induced by the synthetic anticancer agent, etoposide (Tsuda et al., 2001), demonstrating the ability of this inhibitor to suppress the extrinsic apoptotic pathway. This suggests that the apoptotic event observed during the first 24 hours after nephrectomy may be mediated by extrinsic signals. However, since M50054 does not directly bind Caspase 3 (Tsuda et al., 2001), we cannot eliminate other potential mechanisms that may be blocked by M50054 upstream of Caspase 3 activation, such as Granzyme B activity (Creagh et al., 2003) or mitochondrial outer membrane permeabilization (MOMP)-mediated apoptosis (Elmore, 2007).

Notably, immunostaining for Fas receptor was unsuccessful in pre-metamorphic *X. laevis* tadpoles (prior to NF stage 56) (Mangurian et al., 1998), so it is unlikely that this death receptor is involved in the apoptotic event during pronephric regeneration in NF stage 37-41 (0-1 dpn) tadpoles. An alternate death receptor could be involved, but unfortunately very little is known about the apoptotic pathways utilized by tadpoles. Further studies are therefore needed to elucidate the mechanistic induction of apoptosis in this kidney regeneration event.

Since M50054 successfully blocked activation of Caspase 3 in our renal repair model (Figure 3.1B), we used this pharmacological inhibitor to examine the functional role of apoptosis in tubule regeneration. M50054-exposure from 0-1 dpn dramatically enhanced the number of tadpoles that were able to regenerate proximal tubules by 21 dpn (Figure 3.2), suggesting that this early cell death response may be limiting the regenerative potential of pronephric proximal tubules. Further investigation is necessary to confirm this hypothesis and to determine the mechanism by which cell death immediately following injury can suppress regeneration. However, based on the temporal parameters of apoptosis in this system (early induction and brief expression) (Caine and McLaughlin, 2013), we can speculate on the conditions that would require apoptosis and potentially suppress tubule regeneration.

Immediately following nephrectomy, the expression of active Caspase 3 is a likely indicator of dying cells that were affected by the surgery. This cellular population may include damaged epithelial (skin) and endothelial (capillary) cells, and almost certainly includes un-excised proximal tubule cells, which have been shown to be particularly susceptible to apoptosis in several kidney disease models (Havasi and Borkan, 2011; Lelongt and Ronco, 2003). This sensitivity is primarily due to a loss of cell matrix and cell-cell adhesion (Nony et al., 2001; Bergin et al., 2000). In addition, apoptosis induced by tissue injury stimulates an immediate and rapid immune response to neutralize damage and initiate repair (Rock and Kono, 2008). However, this acute inflammatory response is fairly indiscriminate and can damage normal tissues, potentially exacerbating the loss of renal cells.



Importantly, unrestricted death of unexcised proximal tubules may result in severely diminished regenerative capacity in the pronephric kidney. This hypothesis is supported by recent evidence that complete removal of proximal tubules in *X. laevis* tadpoles correlates to an absence of tubule regeneration (Caine and McLaughlin, 2013). A similar requirement for surviving tubule cells in renal restoration has also been observed in several mammalian injury models (Humphreys et al., 2008; Kays and Schnellmann, 1995; Nadasdy et al., 1995; Witzgall et al., 1994; Wallin et al., 1992; Kovacs et al., 1982; Venkatachalam et al., 1978; Cuppage et al., 1972). It is likely then that the cumulative loss of tubule cells during damage and inflammation may be an insurmountable obstacle for a large percentage of nephrectomized tadpoles to overcome. Thus, if detectable apoptosis in the renal area after nephrectomy is an indicator of excessive loss of tubule cells, then the trends observed in this study would suggest that suppression of this cell death early in repair is sufficient to allow three times as many individuals to regenerate pronephric kidneys. Future studies should focus on identifying the cells that undergo apoptosis during this 0-1 dpn period, to confirm their proximal tubule identity.

It is also possible that turn-over of immune cells, such as neutrophils and macrophages (Savill, 1997) may account for some of the apoptotic activity being detected in the kidney. This possibility is strengthened by the fact that *Xenopus* tadpoles rely exclusively on their innate immunity to offset the effects of tissue damage (Tomlinson et al., 2008; Smith et al., 2002). Additionally, because dying cells are usually phagocytized and degraded very quickly, the ability to detect even small amounts of apoptosis may be an indicator of a greater amount of cell death (Savill, 1994). Thus, detection of active

Caspase 3 in unexposed tadpoles at 1 dpn (Figure 3.1B, black bar), and in tadpoles examined as late as 2 dpn (Caine and McLaughlin, 2013), would suggest that the initial inflammatory response after nephrectomy is prolonged in some tadpoles more than others. By comparison, wound closure at the damaged site occurs within 12- 18 hours (data not shown). Protracted inflammation may be detrimental to the repair microenvironment and could result in differential abilities to successfully regenerate.

It would be useful to investigate the correlation between the extent of cell death and the success of regeneration in pronephric repair. While this has been impossible thus far as tadpoles are euthanized prior to immunohistochemical detection of apoptosis, it may be possible moving forward to use injectable *in vivo* assays for detecting apoptosis. For example, the non-toxic NIR FLIVO system (Neuromics), which is imaged in the near-infrared light spectrum (Rao et al., 2007), could be used to directly compare the level of apoptosis at 1 dpn with the regenerative success at 21 dpn in individual tadpoles followed over the course of the 3 week experiment.

The wound healing events discussed above may also involve the activity of MMP-9, which has previously been implicated in the modulation of the inflammatory response during renal damage (Chromek et al., 2004; Gobe and Buttyan, 2002; Savill, 1997) and in the mediation of apoptosis in the kidney (Bengatta et al., 2009). It is possible that the *Xmmp-9* expression previously described (Caine and McLaughlin, 2013) is indicative of a role for the active enzyme in the inflammatory processes in the *Xenopus* system.

Interestingly, MMP-9 has recently been found to actively participate in the innate immune response in fish (Chadzinska et al., 2008). Although a specific

immunomodulatory role for MMP-9 has not (yet) been described in *Xenopus laevis* larvae, expression of this gene is upregulated within 3-6 hours of injury in several tadpole organs including: lens, skin, hind limb buds (Carinato et al., 2000); pronephric kidney (Caine and McLaughlin, 2013); somitic muscle, gut, and tail (Supplemental Figure S3.1). This rapid and indiscriminate response to damage is highly suggestive of a role for MMP-9 in the early stages of wound healing, when mobilization of immune cells would be occurring. To investigate the function of early *Xmmp-9* expression in proximal tubule regeneration, we inhibited the activity of the XMMP-9 protein from 0-1 dpn via exposure to synthetic inhibitors, GM6001 or SB-3CT.

MMP proteolytic activity is inhibited by the binding of the GM6001 hydroxamic moiety to the catalytic zinc (Balakrishnan et al., 2006), which is found in the active site of all MMPs (Springman et al., 1990). GM6001 primarily targets MMP-1 and MMP-9, and also shows affinity for MMP-2, MMP-3, and MMP-8 (Galardy et al., 1994b); however of the 11 MMPs previously identified in *X. laevis* (Harrison et al., 2004), only MMP-9, MMP-11, and MMP-13 have been shown to be expressed during early tadpoles stages (Carinato et al., 2000; Damjanovski et al., 2000). Therefore, in this system (NF stage 37/38 tadpoles) both GM6001 and the MMP-2/MMP-9 inhibitor SB-3CT, specifically block MMP-9 activity.

In pronephric kidney repair, GM6001-inhibition of XMMP-9 activity from 0-1 dpn significantly reduced the regenerative success of nephrectomized tadpoles (Figure 3.3A), indicating that early XMMP-9 activity is critical for the regeneration of pronephric proximal tubules. A similar trend was detected after 0-1 dpn exposure to SB-3CT (Figure 3.3B), however the variance observed with this inhibitor precluded statistical

significance among the treatment groups. While variation in regenerative behavior is thought to be inherent to different batches of *X. laevis* tadpoles (Slack et al., 2004), it is also possible that the variability observed in this study is attributable to the biochemical nature of the SB-3CT/MMP-9 complex.

Whereas GM6001 is a reversible inhibitor (Saghatelian et al., 2004), SB-3CT is a slow binding, mechanism-based inhibitor (Krüger et al., 2005; Brown et al., 2000), which produces a “suicide” or irreversible bond with the target MMP protein (Gu et al., 2005) by altering the conformation of the active site and returning it to a more zymogen-like shape (Kleifeld et al., 2001). Therefore, rinsing the inhibitor out of the system at 1 dpn may not be as effective or uniform among SB-3CT exposed tadpoles, which may introduce slight variability in exposure lengths among individuals. This inconsistency may be sufficient to influence the regenerative success assessed at 21 dpn. For this reason, we are currently confirming these results with a third inhibitor, which binds reversibly and is specific to MMP-9.

Additionally, the percentage of successful regenerates in the 0.06% DMSO-exposed group (vehicle control for SB-3CT), also showed a dramatic decrease (Figure 3.3B). While not statistically significant, this trend was concerning as it may also suggest that the results observed in the SB-3CT-exposed group may be influenced by some toxicity or mechanistic effect in combination with DMSO. Interestingly, it appears as though SB-3CT may be modulating the effects of DMSO in this system however it is unclear how these interactions may be affecting renal regeneration. A similar effect on DMSO has been seen previously with a different pharmaceutical agent (Hallare et al.,

2004), emphasizing the need to perform adequate vehicle controls in studies with pharmaceuticals and organic solvents.

Despite these limitations with SB-3CT, it is clear from the GM6001 exposure studies that XMMP-9 has an inhibitory effect on proximal tubule regeneration. Similar trends have been described in *in vivo* studies in mammalian kidneys, where MMP-9 deficiency has led to compromised renal repair due to excessive ECM deposition (Chromek et al., 2003; Lelongt et al., 2001a; González-Avila et al., 1998). However, we did not detect any histological evidence of excessive matrix assembly in tadpoles exposed to either MMP-9 inhibitor. Currently, we are attempting to confirm this via immunohistochemical detection against a panel of known ECM markers.

A more likely role for XMMP-9 is the modification of the ECM in the creation of a microenvironment permissive for regeneration. A similar role for MMPs, including MMP-9, has been described in the creation of the permissive environment for retina regeneration in the MRL (regenerative) mouse strain (Tucker et al., 2008). Moreover a delicate balance between MMP-9 and its endogenous inhibitor, Tissue inhibitor of metalloproteinase 1 (TIMP-1), has been implicated in the promotion or repair (Bellayr et al., 2009) and the reduction of scarring (Chromek et al., 2003) in two mammalian models of kidney disease.

To confirm a similar role of XMMP-9, it is essential to identify the cellular source(s) of the active enzyme during renal repair. In this study, larvae are nephrectomized at early tadpole stages (NF stage 37/38), at a time when tadpole immunity is limited to the production of myeloid cell types, such as macrophages and neutrophils (Robert and Ohta, 2009). These inflammatory cells are known producers of

MMP-9 (Kunugi et al., 2011; Heber-Katz, 2004; La Fleur et al., 1996) and so are likely candidates for the secretion of XMMP-9 during early stages of pronephric regeneration in *X. laevis*. In support of this, the expression profile of XMMP-9 (Supplemental Figure 3.2) detected in our system closely resembles expression of *XPOX2*, a peroxidase family member that has been used to detect myeloid cells in *X. laevis* during embryonic development and throughout their migration in tadpole stages (Smith et al., 2002). Direct co-expression studies with *Xmmp-9* and *XPOX2* would be useful to confirm this cellular identity. In addition, if XMMP-9 activity is produced by cells of the immune system in *Xenopus*, it would be interesting to observe the effects of introducing immunosuppressant drugs to this system. A recent study in *Xenopus* tadpoles has successfully used a panel of these inhibitors to elucidate the role of the immune system in tail regeneration (Fukazawa et al., 2009). A similar endeavor could be undertaken with the kidney to determine the effects of immunosuppression on both the levels of active XMMP-9 enzyme present after nephrectomy, and the regenerative capacity in the proximal tubules.

Since MMP-9 has also been shown to play a protective, anti-apoptotic role in murine proximal tubule repair (Bengatta et al., 2009), we investigated whether the inhibition of tubule regeneration in our system was due to a loss of a similar function of XMMP-9. While there was a slight increase in the percentage of GM6001-inhibited tadpoles with increased levels of ipsilateral active Caspase-3 at 1 dpn, this trend was not significantly different from the vehicle-exposed or unexposed treatment groups (Figure 3C). Additionally, no difference was found in the average number of active Caspase 3-positive cells in treatments (Figure 3.3D). Therefore it does not appear that XMMP-9 functions to mitigate apoptosis during early stages of pronephric repair.

This result may be explained by the fact that the anti-apoptotic role described in mice involves apical expression of MMP-9 in polarized proximal tubule cells that are already comprised in a lumenized structure (Bengatta et al., 2009). At 0-1 dpn, surviving proximal tubules cells in nephrectomized *X. laevis* tadpoles do appear to retain cell polarity as evidenced by expression of the apical membrane protein 3G8, however these cells comprise fragmented renal tissue that lack an organized lumen (Caine and McLaughlin, 2013). Additionally, the ipsilateral XMMP-9 expression observed in tadpoles at 1 dpn is not localized to apical cell membranes, but rather is very diffuse and punctate in the injured renal area (Supplemental Figure S3.2). However, these data do not rule out the possibility that XMMP-9 could play an anti-apoptotic role later in the regenerative process when a more organized, restored tubule structure may be present. For this reason, we are extending our loss-of-function assays to investigate whether such a role for XMMP-9 may exist during the 5-7 dpn phase of *Xmmp-9* upregulation (Caine and McLaughlin, 2013).

While the specific function of XMMP-9 during pronephric tubule regeneration is still a mystery, this study demonstrates that its proteolytic activity strongly influences the success of tubule restoration. Further studies are therefore required in order to gain a complete understanding of this phenomenon.

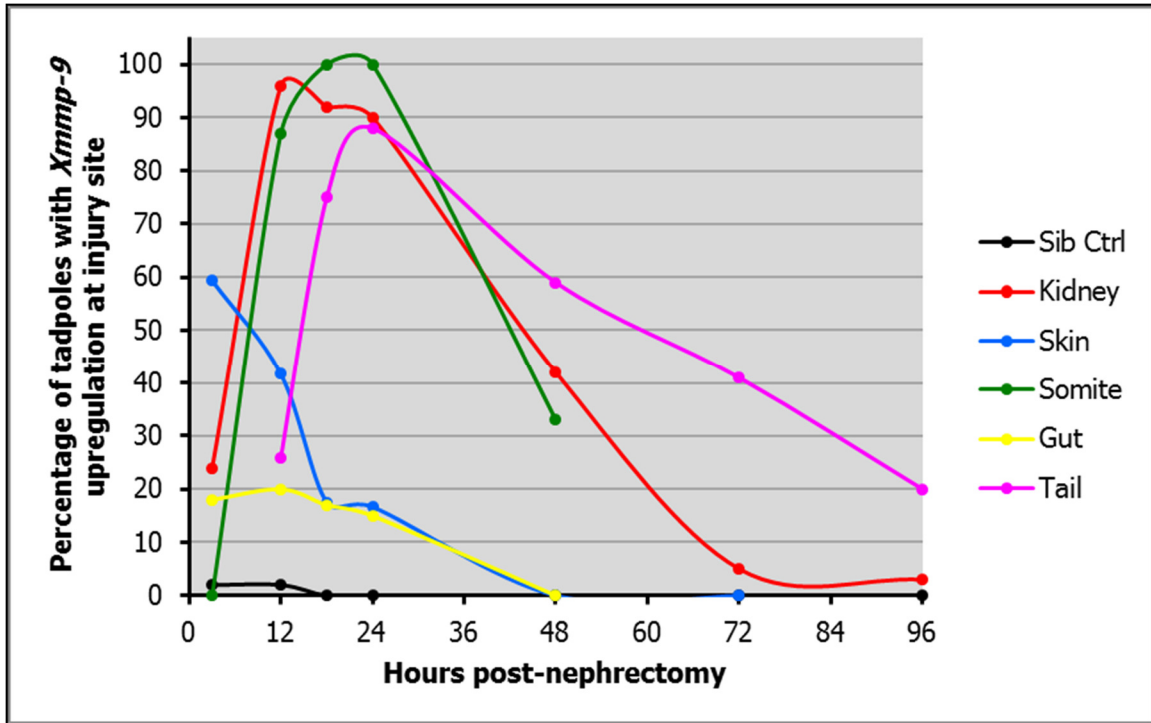
## **ACKNOWLEDGMENTS**

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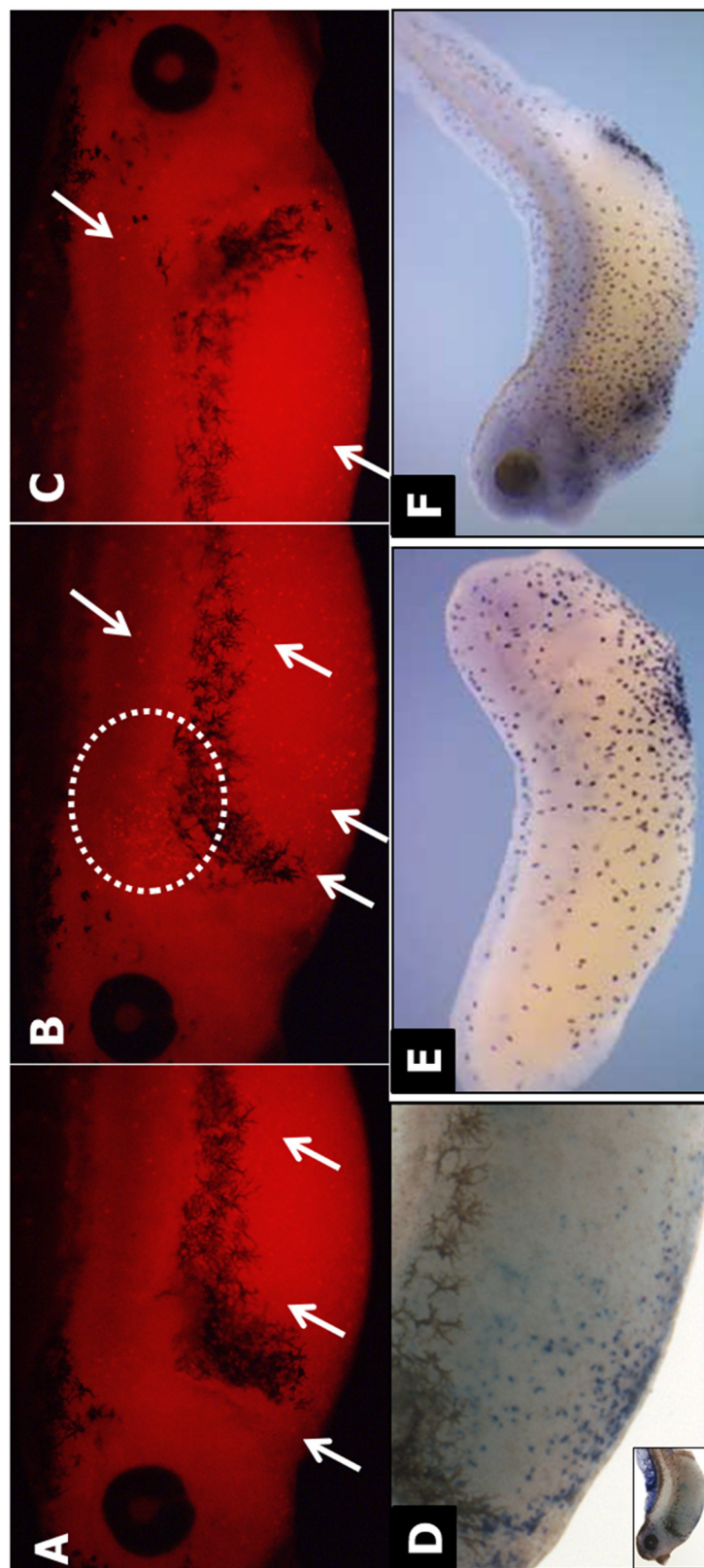
Lewis for assistance with statistical analysis. The authors are also grateful to the members of the McLaughlin lab, the Levin lab and the regeneration community for many useful discussions. This work was generously supported by NSF grant IOS-0843355 (K.A.M.), and the Tufts Summer Scholar's Program (Z.M.).



### SUPPLEMENTAL FIGURES FOR CHAPTER 3



**Supplemental Figure S3.1 *Xmmp-9* expression in injured tissues in *Xenopus laevis*.** Upregulation of *Xmmp-9* is detected within the first few hours of damage in all injured tissues examined. Sib Ctrl, unmanipulated sibling controls; neph, nephrectomized kidneys; skin, incision along lateral flank; somite, somite puncture; gut, gut puncture; tail, amputated tails.



**Supplemental Figure S3.2 XMMP-9 protein and transcript expression patterns are comparable to myeloid cell gene expression patterns.** In unoperated control tadpoles (A) XMMP-9 protein is expressed in a punctate pattern in several areas in the tadpole, especially in the antero-ventral regions and the tadpole flank (white arrows). XMMP-9-positive cells are also seen in the ipsilateral wound area (white outline, B) but not on the contralateral side (C) of nephrectomized tadpoles. The punctate expression and spatial arrangement of these XMMP-9-expressing cells, as well as cells expressing the *Xmmp-9* transcript (D) as seen on the ventral side of a stage 38 tadpole (D, inset) resemble the expression of *XPOX2* (E) and *XLURP-1* (F), which are known markers of myeloid cells in *Xenopus* (Smith et al., 2002). Panels E and F modified from Smith et al., 2002.

## **CHAPTER FOUR**

### **The dualistic roles of Matrix metalloproteinase-9 in pronephric kidney regeneration**

**Data contributions to the chapter:** I contributed data to all figures, with the exception of Figure 4.3B, which was contributed by Zachary Michel. I performed statistical analyses on all figures.

In the previous chapters we established *Xenopus laevis* tadpoles as a model for studying kidney regeneration and described the biphasic expression of *Xmmp-9* during the first week of repair (Chapter 2). Through loss-of-function analyses, we determined that the first phase of XMMP-9 activity (0-1 day post-nephrectomy) promotes tubule regeneration and postulated that this activity may be contributing to the creation of a permissive regenerative environment. Based on the spatial and temporal characteristics of the *Xmmp-9* transcript expression, we also suggested that the cells producing this protease at the wound site are members of the tadpole innate immune system, most likely of the myeloid lineage (Chapter 3). In this chapter (Chapter 4), we expand our knowledge of the role(s) of XMMP-9 during the first week after nephrectomy, by inhibiting protein activity during the second phase (5-7 dpn), and during both phases of expression (0-1 and 5-7 dpn). Our preliminary results support the hypothesis that early XMMP-9 activity promotes regeneration, and reveals an inhibitory role of this proteolytic activity during the second phase. Chapter 4 also suggests potential mechanisms by which these dualistic roles of XMMP-9 may regulate pronephric regeneration.

## **ABSTRACT**

Pronephric proximal tubules are able to regenerate after dramatic tissue loss caused by nephrectomy. However, the mechanisms that regulate this regenerative event are incompletely understood. Previously we have shown that *Xmmp-9*, a member of the matrix metalloproteinase family of extracellular matrix remodelers, is expressed during the proximal tubule regeneration process at very specific windows of repair. Loss-of-

function studies demonstrate an inhibitory role for the XMMP-9 enzyme during the later of these two phases of expression, and support a stimulatory role for the earlier phase on tubule regeneration. These conclusions implicate specific windows of MMP-9 activity as potential targets for therapeutically enhancing the reparability of renal proximal tubules.

## INTRODUCTION

Mammalian kidneys develop through three distinct forms: the embryonic pronephros, the mesonephros (intermediate form) and the terminal metanephros (Saxén, 1987). Severe damage to metanephric renal structures often results in fibrosis or renal scarring (Bellayr et al., 2009; Liu et al., 2009; Chromek et al., 2003), which destroys the structural integrity of the organ, diminishes renal function, and can lead to irreversible conditions such as end-stage renal disease (Wang et al., 2010). Pronephric (Caine and McLaughlin, 2013) and some mesonephric kidneys (Diep et al., 2011; Watanabe et al., 2009; Elger et al., 2003), however, have a greater capacity for repair unaccompanied by fibrosis.

Among these is the pronephric kidney found in the larvae of the amphibian model organism, *Xenopus laevis*. Recently it has been shown that tadpoles possess the ability to regenerate specific segments of the pronephric kidneys called proximal tubules (Caine and McLaughlin, 2013). These segments are analogous to proximal tubules found in more complex mammalian metanephric kidneys (Raciti et al., 2008) and as such may provide a powerful model for discovering potential repair capacities innate to proximal tubules. Investigating the roles of conserved proteins in the pronephric and metanephric

repair pathways would contribute to distinguishing between species-specific adaptations in kidney repair mechanisms.

A suitable candidate for this type of research is the matrix metalloproteinase, MMP-9, which is upregulated after nephrectomy in both pronephric (Caine and McLaughlin, 2013) and metanephric kidneys (Liu et al., 2009). It was recently shown that XMMP-9 activity during the first day after nephrectomy in *Xenopus laevis* promotes regeneration in pronephric kidneys (Caine et al., *in preparation*). However it is unknown what role this enzyme may be playing later in repair, at 5-7 days post-nephrectomy (dpn), which coincides with another established period of *Xmmp-9* gene expression (Caine and McLaughlin, 2013).

To investigate this, we examined the ability of *X. laevis* tadpoles to regenerate proximal tubules after loss of XMMP-9 activity during this specific 5-7 dpn window of repair. Our results indicate that *Xmmp-9* upregulation during this stage of repair may attenuate the regenerative potential of tadpole proximal tubules. Additionally, we investigate the effect of blocking enzyme activity during both phases of repair (0-1 and 5-7 dpn) and the results of these studies support an early (0-1 dpn) stimulatory role and a later (5-7 dpn) inhibitory role for XMMP-9 in renal regeneration. These dualistic roles of MMP-9 at different phases of repair may be useful therapeutic targets for enhancing the regenerative capacity of mammalian metanephric kidneys.

## EXPERIMENTAL PROCEDURES

### *Xenopus laevis* Tadpoles

All experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals and were approved via the Institutional Animal Care and Use Committee (IACUC) at Tufts University. *Xenopus laevis* tadpoles were obtained via a standard *in vitro* fertilization protocol (Caine and McLaughlin, 2013), staged according to Nieuwkoop and Faber (NF, 1994), and reared in 0.1X Marc's Modified Ringer's solution (MMR; 10 mM NaCl, 0.2 mM KCl, 0.1 mM CaCl<sub>2</sub>, 0.2 mM MgCl<sub>2</sub>, 0.5 mM HEPES, 1  $\mu$ M EDTA, pH 7.4) (modified from Kay and Peng, 1991).

### *Partial Unilateral Pronephrectomy*

NF stage 37/38 tadpoles were anaesthetized with 0.04% tricaine in 0.1X MMR, and pronephric proximal tubules were excised as previously described (Caine and McLaughlin, 2013). Stage-matched/sibling damage control (poked multiple times through the skin into the left-side renal region) and unoperated control tadpoles were also collected for each assay. Nephrectomy/damage was always performed in the left (ipsilateral) kidney. NF stage 48 and older tadpoles were fed daily with 0.008% Sera Micron powdered growth food in 0.1X MMR. At the desired time-point (hours or days post-nephrectomy), tadpoles were euthanized with tricaine, fixed for one hour at room temperature in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO<sub>4</sub>, 3.7 % formaldehyde), rinsed in 1X phosphate buffered saline (PBS), and gradually dehydrated to 100% methanol for storage at -20°C.

### *Inhibitor Exposures*

*Xmmp-9* activity was inhibited with either 30 $\mu$ M GM6001 (Calbiochem) or 10 $\mu$ M SB-3CT (MMP-2/MMP-9 Inhibitor IV; Calbiochem or Santa Cruz Biotechnology). Inhibitor stocks were prepared in DMSO and all exposures were conducted at the indicated concentrations by dissolution in 0.1X MMR. “Single” 5-7 dpn exposures were performed by transferring nephrectomized tadpoles to the appropriate exposure medium at 5dpn and refreshing the exposure medium at 6 dpn. Tadpoles were then transferred to new dishes and rinsed three times with 0.1X MMR and reared in 0.1X MMR for the completion of the experiment (21 dpn). “Double” exposures were also performed from 0-1 and 5-7 dpn. Since *Xmmp-9* expression is detectable starting at 3 hours (Caine and McLaughlin, 2013), individual tadpoles were transferred to exposure media within 2 hours of tubule excision. This 0-1 dpn exposure was terminated by transferring larvae to new dishes and rinsing three times with fresh 0.1X MMR. Tadpoles were then re-exposed from 5-7 dpn as described above. To assess the effects of inhibitors on tadpole development, behavior and/or mortality, experiments were also performed on unnephrectomized sibling controls.

### *Assessment of Proximal Tubule Regeneration*

Regenerative success was determined by calculating the percentage of tadpoles from each treatment (unexposed, vehicle-control, inhibitor-exposed) with coiled, 3G8-positive proximal tubules (normal tubule phenotype) at 21 dpn. 3G8 protein expression was detected by whole-mount immunohistochemistry as previously described (Caine and McLaughlin, 2013) using anti-3G8 primary antibody (generously provided by Elizabeth



Jones, Ph.D.), and alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibodies, which was developed with 5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium (1:600 BCIP/1:600 NBT; Roche Diagnostics). At 21 dpn, it is difficult to distinguish between true regenerated kidneys and inadequately operated kidneys that have retained normal tubule phenotype for the extent of the assay. Thus, to account for the proportion of each cohort that may have been insufficiently nephrectomized, representative tadpoles were also collected at 1 dpn and assayed for 3G8 expression. The percentage of tadpoles on 21 dpn that successfully regenerated proximal tubules was calculated for each group/replicate by correcting for the percent of tadpoles that retained full 3G8 expression at 1 dpn.

## RESULTS

### *XMMP-9 activity from 5-7 dpn may impede proximal tubule regeneration*

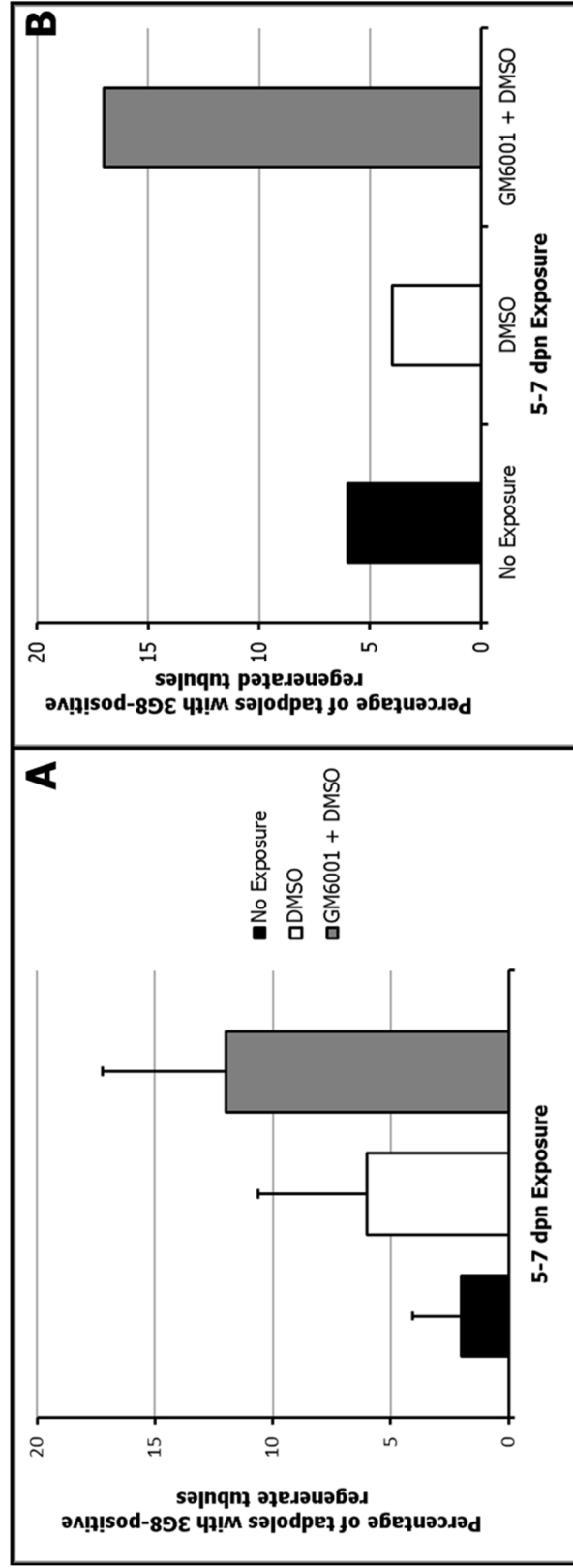
To examine the role of the regeneration-specific pattern of *Xmmp-9* expression observed on the ipsilateral side of nephrectomized tadpoles at 5-7 days post-surgery (Caine and McLaughlin, 2013), we pharmacologically inhibited XMMP-9 activity with one of two known inhibitors, GM6001 or SB-3CT. No unusual behavior or gross morphological effects were observed in nephrectomized tadpoles or unoperated siblings exposed to the broad-spectrum MMP inhibitor, GM6001; the MMP-2/MMP-9 inhibitor, SB-3CT; or the vehicle DMSO during the course of these experiments.

Exposure to GM6001 correlated to an increase in the percentage of tadpoles with regenerated (fully, coiled, 3G8-positive) proximal tubules at 21 dpn as compared to both

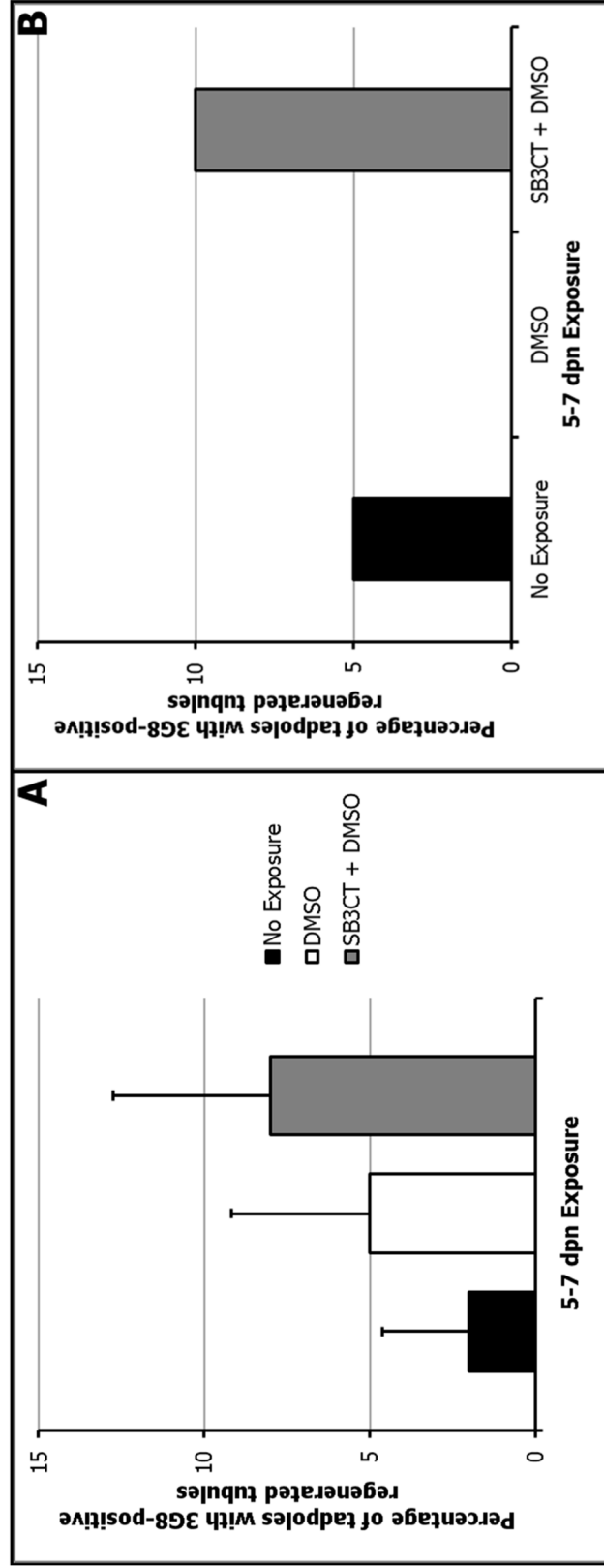
vehicle-exposed (2-fold) and unexposed controls (6-fold; Figure 4.1A). While the differences among these treatment groups is not statistically significant (Kruskal-Wallis test,  $p = 0.5$ ), the trend is suggestive of an inhibitory role for XMMP-9 during this period of tubule regeneration. This hypothesis is supported by the trend observed after SB-3CT exposure, which also resulted in a greater number of regenerates at 21 dpn (Figure 4.2A). Unfortunately, the variability in these data precluded any significant differences to be calculated among the treatment groups.

Typically, about 17% of nephrectomized tadpoles are able to fully regenerate proximal tubules in 3 weeks (Caine and McLaughlin, 2013). However, the percentage of unexposed tadpoles with regenerated tubules was unusually low (2%) in both inhibitor studies (Figures 4.1A and 4.2A, black bars). This was due in large part to most replicates having no regenerates present in the unexposed treatment group. Since inhibition of XMMP-9 from 0-1 dpn has been found to suppress tubule regeneration when compared to unexposed siblings (Chapter 3, Caine et al., *in preparation*), we were concerned that replicates with 0% regeneration in unexposed tadpoles in the present studies would bias our analysis of the 5-7 dpn exposures, allowing us to only detect an increase in regeneration success. With no regeneration in the unexposed treatment group, we would be unable to distinguish between a reduction in the regenerative success and no effect on exposed tadpoles.

For this reason, we eliminated cohorts of nephrectomized tadpoles with no regeneration in the unexposed tadpole treatment group at 21 dpn and reanalyzed the trends. Unfortunately only one replicate from each inhibitor study was available for this re-analysis, however these replicates both confirmed that inhibition of XMMP-9 activity



**Figure 4.1 Inhibition of XMMP-9 activity promotes tubule regeneration in *Xenopus laevis* tadpoles.** Nephrectomized tadpoles were exposed to 30 $\mu$ M GM6001 (in 0.012% DMSO) from 5-7 days post-nephrectomy (dpn) and then assayed at 21 dpn for the presence of fully coiled, regenerated tubules via expression of the differentiated proximal tubule protein 3G8 on the ipsilateral (operated) side. (A) Error bars indicate standard error among 3 replicates, n=41-53. (B) Single replicate of GM6001-exposure study, n=16-25.



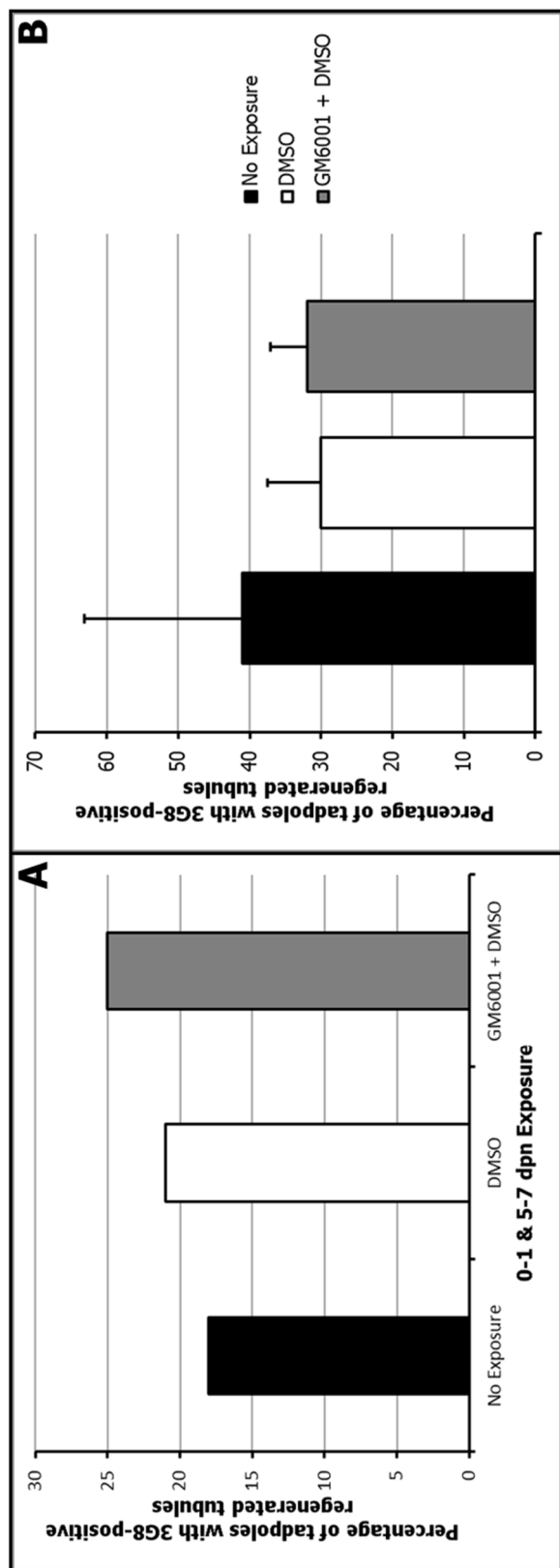
**Figure 4.2 SB-3CT inhibition of XMMP-9 activity promotes tubule regeneration in *Xenopus laevis* tadpoles.** Nephrectomized tadpoles were exposed to 10 $\mu$ M SB-3CT (in 0.06% DMSO) from 5-7 days post-nephrectomy (dpn) and then assayed at 21 dpn for the presence of fully coiled, regenerated tubules via expression of the differentiated proximal tubule protein 3G8 on the ipsilateral (operated) side. (A) Error bars indicate standard error among 2 replicates, n=26-55. (B) Single replicate of SB-3CT-exposure study.

from 5-7 dpn is correlated to increased proximal tubule regeneration at 21 dpn (Figures 4.1B and 4.2B). The persistence of these trends strongly suggests that XMMP-9 activity during the 5-7 dpn window has an inhibitory effect on tubule regeneration.

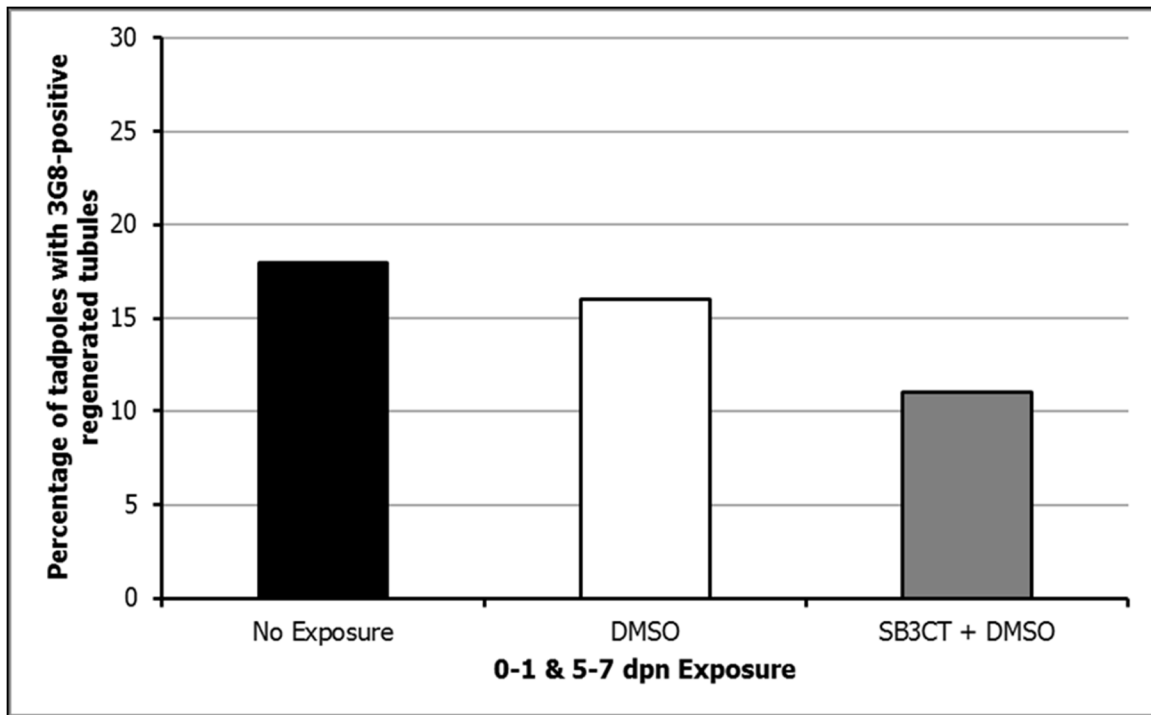
*Double exposures may confirm an inhibitory role for XMMP-9 activity at 5-7 dpn*

Since XMMP-9 activity from 0-1 dpn appears to promote renal regeneration (Chapter 3, Caine et al., *in preparation*) and activity from 5-7 dpn may inhibit this process (Figures 4.1 and 4.2), we were interested in determining the overall effect of blocking XMMP-9 function during both periods of repair. In the pilot study with the double (0-1 and 5-7 dpn) GM6001 exposure, a mere 1.2-fold increase in regeneration is seen in the inhibitor-exposed group relative to the vehicle-exposed group (Figure 4.3A). Compared to the 4-fold increase over the vehicle-exposed group observed in the single (5-7 dpn) GM6001 exposure (Figure 4.2B), the preliminary results of the double exposure indicate no net effect on the number of tadpoles able to regenerate proximal tubules by 21 dpn (Figure 4.3A). This study was repeated in triplicate by a second experimenter who also saw no net effect on the percentage of regenerates at 21 dpn (Kruskal-Wallis test,  $p = 0.74$ ; Figure 4.3B).

A preliminary double exposure study was also performed with the SB-3CT inhibitor, which similarly revealed no overall effect on the percentage of tadpoles with regenerated tubules (Figure 4.4). Taken together, the results of these double exposure studies suggest that blocking XMMP-9 activity from 5-7 dpn may compensate for the inhibitory effects observed after the loss of XMMP-9 activity early (0-1 dpn) in the regenerative process (Chapter 3, Caine et al., *in preparation*).



**Figure 4.3 Double GM6001 inhibition of XMMP-9 activity has no net effect on tubule regeneration in *Xenopus laevis* tadpoles.** Nephrectomized tadpoles were exposed to 30 $\mu$ M GM6001 (in 0.012% DMSO) from 0-1 day then again from 5-7 days post-nephrectomy (dpn), then assayed at 21 dpn for the presence of fully coiled, regenerated tubules via expression of the differentiated proximal tubule protein 3G8 on the ipsilateral (operated) side. (A) Single replicate/pilot study of double GM6001 exposure study. (B) Error bars indicate standard error among 3 replicates, n=33-34.



**Figure 4.4 Double SB-3CT inhibition of XMMP-9 activity has no net effect on tubule regeneration in *Xenopus laevis* tadpoles.** Nephrectomized tadpoles were exposed to 10 $\mu$ M SB-3CT (in 0.06% DMSO) from 0-1 day then again from 5-7 days post-nephrectomy (dpn), then assayed at 21 dpn for the presence of fully coiled, regenerated tubules via expression of the differentiated proximal tubule protein 3G8 on the ipsilateral (operated) side. Graph represents a single replicate/pilot study of double SB-3CT exposure study.

## DISCUSSION

The early biphasic expression of *Xmmp-9* observed during the first week after nephrectomy strongly suggests a role(s) for this ECM remodeler in mediating wound healing and inflammatory responses in pronephric proximal tubule regeneration. This hypothesis is supported by the dramatic effects that the loss of XMMP-9 activity has on the outcome of tubule repair. Previously we have shown that inhibiting the early phase of *Xmmp-9* expression (0-1 dpn) significantly reduces the regenerative ability of *X. laevis* tadpoles (Chapter 3, Caine et al., *in preparation*). In these studies, we examined the effects of inhibiting either the later phase (5-7 dpn) or both phases (0-1 and 5-7 dpn), and our preliminary results suggest that the activity of XMMP-9 from 5-7 dpn may be detrimental to the progression of tubule regeneration. While the data supporting this hypothesis is very preliminary, the consistency in the observable trends using two different inhibitors of XMMP-9 is promising (Figures 4.1-4.2), and as such we discuss potential mechanisms that may explain an inhibitory role for XMMP-9 during the 5-7 dpn window of pronephric repair.

The *Xmmp-9* expression pattern at 5-7 dpn appears as individual cells accumulating at the nephrectomy site (shown in Figure 2.5G, Caine and McLaughlin, 2013). These *Xmmp-9*-positive cells are likely members of the myeloid lineage, which function during inflammatory responses coordinated by the tadpole's innate immunity (Robert and Ohta, 2009; Smith et al., 2002). To confirm this cellular identity, it would be useful to perform co-expression studies with *Xmmp-9* transcript (or antibodies against the zymogen) with known markers of myeloid cells in *Xenopus*, such as *XPOX2* or *XLURP-1*



(Smith et al., 2002). Elucidating the source of XMMP-9 production would be informative about the potential roles of this protease in pronephric repair.

Although MMP-9 is known to be secreted by several cell types (Kunugi et al., 2011; Arkell and Jackson, 2003; Atkinson and Senior, 2003; La Fleur et al., 1996), macrophages are the predominant cellular source for secretion of this enzyme during renal fibrosis (Kui Tan et al., 2013; Tan et al., 2010). MMP-9 can degrade Collagen IV in the tubule basement membrane, promoting the epithelial-to-mesenchymal transition of tubular epithelia to myofibroblasts, which is the hallmark of renal scarring in mammalian kidneys (Wang et al., 2010; Yang et al., 2002). However, accumulation of connective tissue in the *Xenopus* system has only been occasionally observed as small bundles of fibrillar-like tissue (resembling a cotton ball), which do not form consistently at the site of injury, and have never been seen beyond 4 dpn (data not shown). Moreover, a distinct lack of fibroproliferation is characteristic of the response to pronephrectomy, even in tadpoles that fail to regenerate tubules. Therefore, it appears that XMMP-9 is not promoting fibroproliferation in this repair pathway, and in fact may be functioning in the area to limit fibrotic accumulation as is seen in other renal models (Bengatta et al., 2009; Liu et al., 2009; Chromek et al., 2003; Lelongt et al., 2001a; González-Avila et al., 1998) and dysfunctional organs (Cabrera et al., 2007).

However, despite this potential anti-fibrotic role of XMMP-9, the results of our GM6001 and SB-3CT exposures at 5-7 dpn suggest that XMMP-9 is having an inhibitory effect on regeneration during this particular window of repair. A likely alternative macrophage-mediated mechanism involving the production of MMP-9 that may be occurring in our system is the promotion of inflammatory infiltration. This detrimental

function of MMPs is notorious in allograft rejection experienced by renal transplant patients (Singh et al., 2010) and has been associated with pathologies in several other tissues (Matsumoto et al., 2009; Gong et al., 2008; Mroczko et al., 2008). GM6001-inhibition of XMMP-9 activity would then prevent this particular inflammatory intrusion during this crucial window of repair, thereby promoting regeneration. In support of this hypothesis, topical application of GM6001 has been shown to prevent infiltration of inflammatory cells in other disease models (Galardy et al., 1994a).

If XMMP-9 is responsible for excessive inflammatory infiltration from 5-7 dpn, this function may be disruptive to the preservation of a permissive microenvironment for pronephric regeneration. In combination with the results of the single exposure inhibitor studies (0-1 dpn or 5-7 dpn), the results of our double inhibitor exposure assays have provided some insight into the nature of such as a permissive environment. Inhibition of XMMP-9 activity from 0-1 dpn suppresses regeneration (Chapter 3, Figure 3.3A; Caine et al., *in preparation*), implying that this activity is necessary for proper repair. In contrast, abolishing XMMP-9 function from 5-7 dpn enhances the regenerative success of tadpoles (Figures 4.1-4.2), while blocking activity during both phases has no net effect (Figure 4.3). Since the net effect of double exposures is not inhibition of regeneration as seen in the 0-1 dpn single exposures, then it is likely that the inhibition of the second phase of XMMP-9 function (5-7 dpn) is sufficient to ameliorate the effects of losing XMMP-9 activity from 0-1 dpn.

Taken together, these results suggest that the permissive microenvironment in renal regeneration is dynamic and adaptable and may be enhanced therapeutically by targeted pharmacological inhibition of deleterious MMP-9 activities. However, the

dualistic roles for MMP-9 illustrate the need to limit therapies to specific windows of repair during which target enzymes exert negative influences in order to avoid unintentionally abating the potentially positive effects of the same enzymes. Additionally, elucidating the downstream effectors of MMP-9 function will be beneficial to tailor these therapies even further. In this way, we can enhance the regenerative capacities of mammalian proximal tubules and stimulate them to behave more like amphibian tubules.

## **ACKNOWLEDGEMENTS**

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## **CHAPTER FIVE**

### **Summary and Conclusions**

As developmental biologists, we are deeply interested in the naissance of tissues and their progression from embryogenesis through to maturation in living systems. As regenerative biologists, we are concerned with understanding how these tissues are able to perceive injury and restore themselves after loss. As kidney researchers, we embrace an appreciation for the formation, growth, maintenance and function of the renal system, methodically explore its components, and contribute to the fields of renal physiology and nephrology. During the course of my research in the McLaughlin laboratory, I recognized the need to endorse all three positions in order to effectively investigate the regeneration of the pronephric kidney in the developing larvae of the amphibian, *Xenopus laevis*. The primary goals of this research were: (1) to determine the regenerative capacity of the pronephric kidney in *X. laevis*, and (2) to use this model organism to elucidate the molecular mechanisms involved in the reparative processes induced by severe renal damage.

To investigate the regenerative capacity in *Xenopus*, we chose to induce proximal tubule damage by unilateral pronephrectomy. Earlier studies utilizing this technique have focused on the response of the unmanipulated, contralateral kidney, which becomes swollen and hypertrophic to compensate for the loss of its counterpart (Scadding and Liversage, 1974; Chopra and Simnett, 1969; Babaeva, 1964; Fox, 1963; Swingle, 1919; Howland, 1916). In our studies, however, the reparability of the injured pronephros was determined, both morphologically/histologically and with the use of molecular tools to characterize the gene expression profiles of cells comprising both the wound site and the regenerate. With these studies, we demonstrate for the first time that *X. laevis* tadpoles are capable of regenerating functional pronephric proximal tubules, and that slightly less

than a fifth of the population (approximately 17%) are able to complete this regenerative process within three weeks of partial nephrectomy. An additional 34% of the population seems to be in various stages of repair at this 21 day post-nephrectomy time-point, which may indicate that these individuals require more time to successfully undergo proximal tubule regeneration.

This variability is the hallmark of most if not all parameters of this regenerative process. No two tadpoles behave the same, and no two tadpoles regenerate the same. For this reason, we can never describe this process in absolutes, and thus rely on percentages of the population to indicate whether our manipulations are influencing the regenerative success of the “average tadpole”. In this way, we can begin to elucidate the mechanism that may *contribute* to successful regeneration, but can never say what will *cause* regeneration.

#### *XMMP-9 in pronephric kidney repair*

*Matrix metalloproteinase-9* is a key component of the amphibian wound healing and regenerative response (Carinato et al., 2000; Yang et al., 1999), and for this reason we chose to examine its expression and function in pronephric proximal tubule repair. This protease, a highly regulated secreted member of the MMP family, has been implicated in several kidney disease models (Caine and McLaughlin, 2013; Kunugi et al., 2011; Wang et al., 2010; Bengatta et al., 2009; Liu et al., 2009; Yao et al., 2009; Chromek et al., 2003; Yang et al., 2002; Lelongt et al., 2001a; González-Avila et al., 1998; McMillan et al., 1996).

The difficulty with studying MMP-9 is that this enzyme has several substrates, both extracellular matrix (ECM) and non-ECM components, and thus the active protein has several potential (and actual) roles in the wound milieu (Bengatta et al., 2009; Legallicier et al., 2001; Lelongt et al., 2001b). MMP-9 has been shown to be involved with both the synthesis and the degradation of the ECM, and can function in both anti-fibrotic and pro-inflammatory contexts. Most of these roles have been described in mammalian metanephric kidneys, which can repopulate denuded basement membranes during acute renal injury (Boti et al., 1982) but experience fibrosis (renal scarring) under chronic conditions (Wang et al., 2010; Liu et al., 2009; McMillan et al., 1996). However the multifaceted (and often unpredictable) nature of MMP-9 in renal repair is likely not limited to re-epithelialization or fibrotic events in mammalian kidneys, as implicated by the results of our studies. Via loss-of-function assays using pharmacological inhibitors, we demonstrate that the biphasic expression of *Xmmp-9* represents two distinct phases of disparate XMMP-9 activity.

During the early phase (0-1 dpn), *Xmmp-9* is expressed in a punctate pattern in cell clusters at the site of injury. The temporal and spatial characteristics of this expression pattern suggest that *Xmmp-9* is being upregulated at the site of nephric injury by cells involved in the innate immunity response to damage. During such an immediate reaction, innate immune cells are likely phagocytizing other cells undergoing apoptosis at the site of damage. This is supported by our detection of an increase in apoptotic cells at the wound site during this 0-1 dpn phase. We speculate that these dying cells represent damaged proximal tubules that were not excised but are no longer comprised of viable cells. It is also likely that some of these apoptotic cells are neutrophils that die during

this inflammatory process. Further investigation is needed to confirm whether the *Xmmp-9*-positive cells are indeed tadpole immunity cells. Co-expression analyses with known markers of *Xenopus* myeloid cells (Smith et al., 2002) as well as immunosuppressant assays that knock-down immune responses (Fukazawa et al., 2009) during this phase of repair would be useful undertakings to this end.

Although the function of XMMP-9 during this window of repair is still unclear, our results indicate that this early enzymatic activity promotes regeneration in this system. It is possible that XMMP-9 may be mitigating the cellular processes that cause fibroproliferation, thereby creating a microenvironment at the wound site that is more permissive for subsequent regeneration. The characteristics of such a microenvironment remain unknown, and thus downstream effects of this *Xmmp-9* expression and XMMP-9 activity need to be investigated further.

The second window of *Xmmp-9* expression, 5-7 dpn, has similar cellular characteristics to the early phase. Expression is punctate and clustered, albeit with comparably attenuated transcript levels (qualitatively assessed via *in situ* hybridization). XMMP-9 may also be produced by innate immune cells during this window of repair, but its function in this capacity appears to be completely different. The results of our inhibitor studies implicate an inhibitory role for this protease during this later phase. Moreover, the inhibition of both phases (0-1 and 5-7 dpn) suggests that the loss of this inhibitory role during the late phase is sufficient to neutralize the negative effects of losing proteolytic activity during the first phase. Taken together, these data suggest that any permissive environment that has been created at the wound site (possibly with the



assistant of 0-1 dpn XMMP-9 activity) may be jeopardized by the proteolytic activity at 5-7 dpn.

One possible explanation of this is the macrophage-mediated inflammatory infiltration observed in several systems (Matsumoto et al., 2009; Gong et al., 2008; Mroczko et al., 2008). Inflammatory infiltration is notoriously damaging and is associated with graft rejection in renal transplant patients (Singh et al., 2010). Macrophage secretion of XMMP-9 for this purpose could destroy critical ECM components that are necessary for providing a physical support system for rebuilding tubules, and/or involved in signaling cascades to induce tubulogenesis. Further investigation is critical to elucidate these potential mechanisms.

Interestingly, the dualistic role for XMMP-9 during the two phases (0-1 dpn and 5-7 dpn) of *Xmmp-9* upregulation observed in this system may account for the low level of regeneration observed in *X. laevis* tadpoles populations (about 17%, Caine and McLaughlin, 2013). While the vast majority of tadpoles display *Xmmp-9* upregulation from 0-1 dpn, a small percentage (about 7%) has no detectable increase in transcript levels. This subpopulation of nephrectomized tadpoles may be comparable to exogenously inhibited tadpoles that have no XMMP-9 activity and fail to regenerate. Related to this, the 50% of the nephrectomized population that re-expresses *Xmmp-9* from 5-7 dpn, may experience the deleterious effects of proteolytic activity suggested by the results of our inhibitor studies and are thus also less competent to regenerate proximal tubules. Taken together, these results demonstrate a need to carefully regulate the timing of *Xmmp-9* expression and by extension, XMMP-9 activity, in order to maximize the regenerative capacity of the tadpole proximal tubule.

However, it is clear that the timing of *Xmmp-9* expression alone cannot sufficiently predict the regenerative success of any give tadpole population. This is unsurprising since it is unlikely that MMP-9 alone can govern an event as multifaceted and extensive (both mechanistically and temporally) as renal regeneration. This metalloproteinase may be essential for preparing the renal permissive microenviroment, an event which has been described in both regenerative pathways (Ingber and Levin, 2007) and cancer progression (Noël et al., 2008). Thus MMP-9 may be particularly critical during the early stages of the renal repair program in vertebrate proximal tubules (in *X. laevis* this may equate to the first 7 days post-nephrectomy).

#### *Regenerative Medicine and Kidney Disease*

Humans, like most mammals, have a very limited regenerative capacity, relying instead on localized wound healing with minimal restoration of lost structures. Tissue regeneration would be an incredibly beneficial therapeutic tool to address the pathology associated with several human diseases, specifically those with current treatments limited to invasive organ/tissue transplant.

While biologists find regeneration to be among some of the most fascinating phenomena worth exploring, our interest in this field is also influenced by the severity and/or prevalence of human disease. Organ regeneration would provide a unique opportunity for replacing damaged tissues with healthy tissues, without the requirement of donor organ transplant. This could either be achieved by stimulating the body to repair damaged organs, or by growing replacement organs in a laboratory to be transplanted in response to irreparable damage. The kidney is one of several organs currently being

investigated by both biologists and tissue engineers for the development of such regenerative therapies (Atala, 2004; Hipp and Atala, 2004).

The Center for Disease Control and Prevention (CDC) reports that one in ten Americans (about 26 million) currently suffer from chronic kidney disease (CKD), while the National Kidney Foundation (NKF) estimates that an additional 350,000 people have end-stage renal disease (ESRD) (NIDDK & NKF). As of February 2013, the United Network for Organ Sharing (UNOS) reported over 95,000 Americans awaiting a kidney transplant (UNOS). In 2012, less than 17,000 patients actually received a transplant (UNOS). These statistics also overlook the tens of thousands of patients diagnosed with acute kidney failure (ARF) each year. Other than organ transplant, dialysis remains the only viable treatment option for both CKD and AKF patients. Over 400,000 Americans rely on this costly and invasive technique each year (NIDDK). The striking inequality between patient number and available treatments illustrates the need for alternative therapies such as regenerative medicine.

Thus, the dualistic roles of MMP-9 described in these studies reveal potential therapeutic targets for enhancing the regenerative capacity of mammalian metanephric kidneys. Such therapies would have to be highly regulated to reduce the risk of inhibiting the protein during a stage of disease progression when it may be playing a protective role. As such, further investigation is critical to understanding exactly how MMP-9 fits into the renal regeneration pathway, and thus determining the role for this protease in the *Xenopus laevis* pronephric model is paramount to understanding how MMP-9 may mediate vertebrate renal repair in general. Since tadpoles are more regenerative animals than mammals, we hope that the role(s) of MMP-9 in this system will reveal important

events in pronephric regeneration that may be induced in the human kidney. Elucidating the cellular mechanisms that regulate these reparative and regenerative events would be beneficial for the development of renal disease therapies, and would also contribute to distinguishing between species-specific adaptations in kidney repair mechanisms.

## **APPENDIX I**

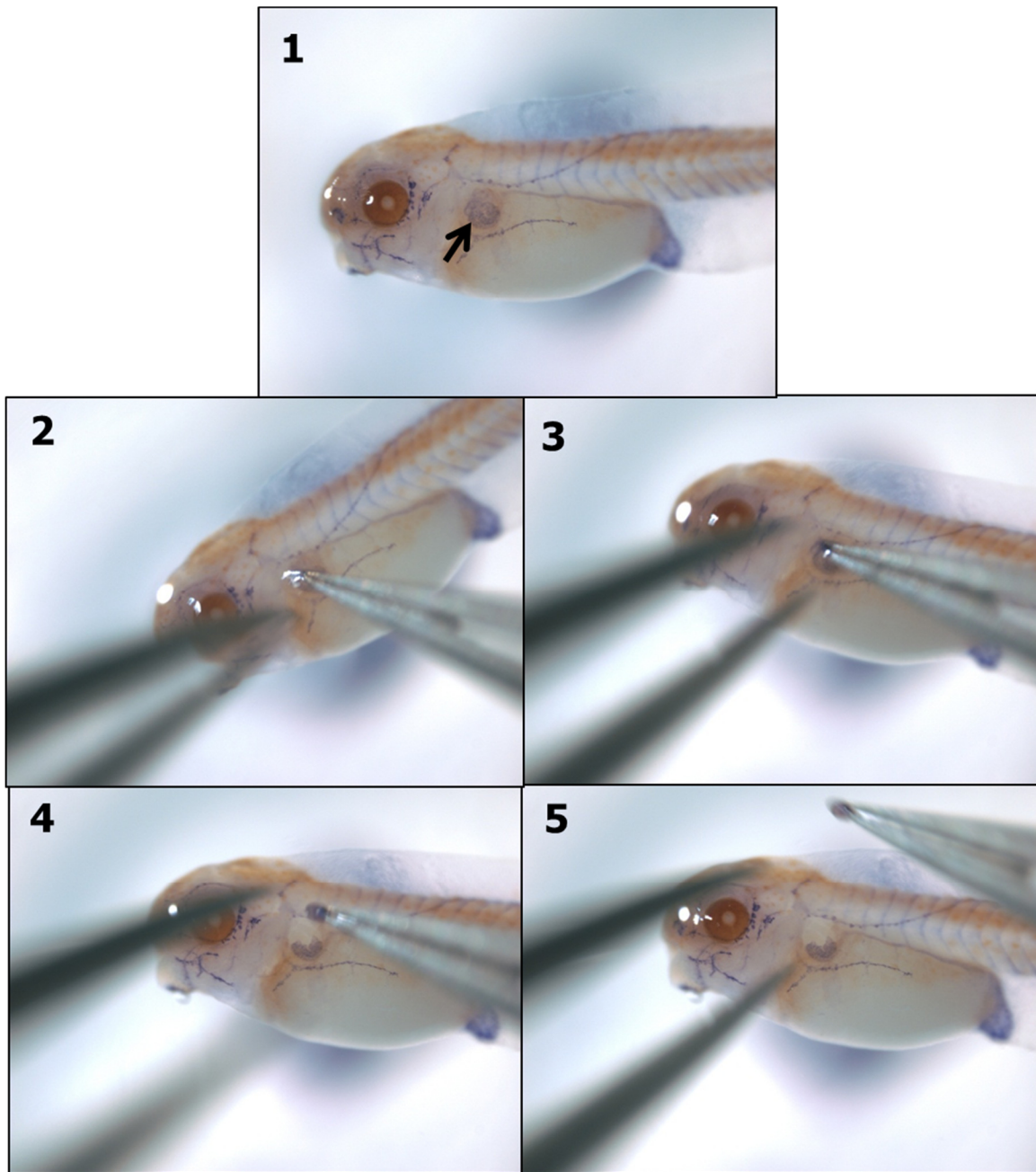
### **Designing the partial unilateral pronephrectomy**

This dissertation (and included manuscripts), contributes a new injury model to the study of renal repair. Below we describe the development of this model and the future improvements that would strengthen the results obtained in studies similar to those described in previous chapters.

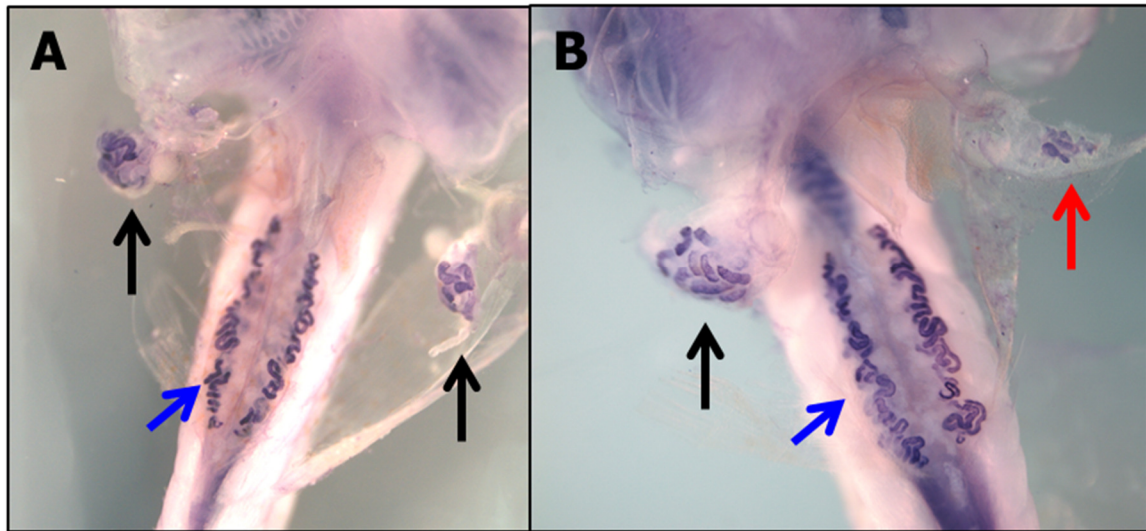
#### *Development of the partial unilateral pronephrectomy protocol*

To investigate the regenerative capacity of *Xenopus laevis* tadpoles, we first needed to select and optimize an injury model to generate tractable and reproducible loss of pronephric tissue. Thus, we developed the partial unilateral pronephrectomy, which involves manual excision of the proximal tubule segments from one pronephros in a tadpole. The specifics of this technique were modeled after the successful removal of the pronephros in larvae of the spotted salamander, *Amblystoma punctatum*, by Ruth B. Howland (Howland, 1916). In this pioneering publication, Howland describes two distinct nephrectomy procedures, both involving the careful incision of skin overlaying the pronephros prior to nephrectomy. Our protocol uses a combination of methods described in these two procedures, and involves a single skin incision cephalad to the pronephros, at the gill slit (Chapter 2, Caine and McLaughlin, 2013) (Figure A1.1).

We have also made similar observations to those discussed by Howland in her original work, such as: bilateral nephrectomy results in massive edema in tadpoles, and ultimately leads to death within a week; unilateral nephrectomy results in hypertrophy of the contralateral kidney; mesonephric development appears to progress normally (Figure A1.2).



**Figure A1.1 Steps in a partial unilateral pronephrectomy.** This NF stage 41 tadpole was euthanized, fixed in formaldehyde and assayed for Na, K ATPase protein to visualize the pronephric tubules, and is used here as a simulation of an anesthetized live tadpole. Steps 1-5 demonstrate the manual excision of a large portion of the tubules. Kidney indicated by arrow (1). Skin is pinched at gill slit and pulled back (2). Tubules are snipped out (3) and discarded (4-5).



**Figure A1.2 Mesonephric and pronephric kidneys in *Xenopus laevis* tadpoles.** The morphology and expression of the protein 3G8 appear normal in mesonephric kidneys (blue arrows) in unoperated control (A) and nephrectomized tadpoles (B) as 21 dpn. Black arrows indicate unoperated control or contralateral kidneys. Red arrow indicates regenerated tubules on ipsilateral side of nephrectomized tadpoles. The gut has been removed to photograph the mesonephric tubules.



Previously, pronephrectomies have been performed in *Rana sylvatica* larvae (Swingle, 1919) as well as in *Xenopus*. However, the pronephrectomy in *Xenopus* was accomplished via cauterization (Chopra and Simnett, 1969), and thus, to our knowledge, no technique involving surgical excision of *Xenopus laevis* pronephroi has ever been performed (or published) prior to ours.

For the studies described in this dissertation, all nephrectomies were performed in NF stage 37/38 tadpoles, at the onset of renal function (Nieuwkoop and Faber, 1994). However, this technique is adjustable and has been used successfully to excise renal tissue at earlier (NF stage 35/35) and later stages (up to NF stage 42) of tadpole development. NF stage 37/38 tadpoles were selected to allow three weeks for regeneration prior to the natural degeneration of the pronephros just before metamorphosis (discussed in Chapter 2, Caine and McLaughlin, 2013). We also preferred to nephrectomize kidneys that were fully differentiated to eliminate confounding effects of disrupting organ development.

Additionally, it was necessary to optimize the conditions for performing the nephrectomy and monitoring tadpoles for the three weeks, since regenerative processes can be influenced by environmental factors such as temperature (Poss et al., 2003). As an ectothermic animal (Gillooly et al., 2001), the tadpole develops more quickly at warmer temperatures, thus individuals raised at room temperature for three weeks post-nephrectomy were significantly older (NF stage 50/51) than those raised at 18°C (NF stage 48). The results of a preliminary study indicate that this developmental difference had no effect on the regenerative success of tadpoles (30% regenerated kidneys in both groups). However, since older tadpoles are notoriously harder to work with when

performing molecular techniques (especially *in situ* hybridization), we decided that it would be more beneficial to raise tadpoles at 18°C.

We were also concerned that at higher temperatures, a faster metabolic rate may affect tadpole mortality after surgery, perhaps due to excessive hemorrhaging. To examine this, we allowed tadpoles to recover from surgery overnight at three temperatures: 14°C, 18°C and room temperature (roughly 22°C). No real differences were observed in the number of deaths or edemas at 1 dpn in any group (data not shown). However, we decided to be cautious and allow tadpoles to recover overnight at 14°C in all future experiments. They are then transferred to 18°C at 1 dpn and raised at this temperature for the remainder of each study.

Lastly, we are aware that the anesthetic tricaine (MS-222), which is used routinely in fish and amphibian laboratories, is also an inhibitor of regeneration (Tseng et al., 2010; Hoffman and Basinger, 1977). However, the concentration used as an anesthetic in our studies is 100-fold lower than the lowest concentration used to block tail regeneration in *Xenopus laevis* tadpoles (Tseng et al., 2010). We also limit the anesthesia time period, to minimize exposure to tricaine as much as possible.

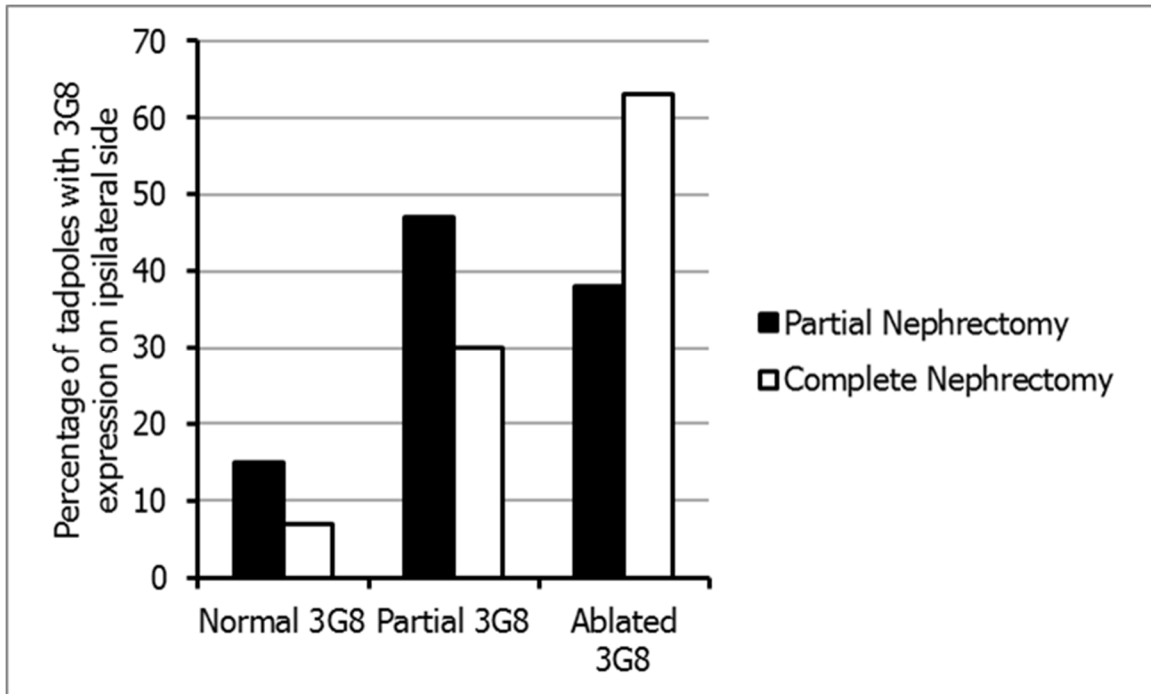
Because pronephroi are approximately a quarter of a millimeter in length (by comparison, NF stage 37/38 tadpoles are about 6 mm long), nephrectomies must be performed under microscopy and with meticulous care. Since the requirements for renal regeneration are unknown, we attempted to minimize confounding variables as much as possible. Thus, tadpoles were euthanized and discarded based on very conservative criteria including prolonged exposure to anesthesia, nicked guts or other damage, and excessive skin removal or duct damage during nephrectomy. These precautions

described above illustrate the efforts we expend to ensure that this protocol is reproducible among surgeries, and among surgeons.

#### *Partial versus complete nephrectomy*

The results of preliminary studies suggested that complete removal of pronephric tubules (as compared to partial removal) correlated more often with an absence of regeneration at 21 dpn (Figure A1.3). Conversely, fewer tadpoles were able to regenerate after “complete” nephrectomies. These data suggest that pre-existing tubule tissue may be an important advantage for successful regeneration. This hypothesis is supported by work from several labs that have demonstrated that surviving proximal epithelial cells reorganize to repopulate tubule basement membranes after injury (Humphreys et al., 2008; Kays and Schnellmann, 1995; Nadasdy et al., 1995; Reimschuessel and Williams, 1995; Witzgall et al., 1994; Nonclercq et al., 1992; Wallin et al., 1992; Kovacs et al., 1982; Venkatachalam et al., 1978; Cuppage et al., 1972). This requirement for pre-existing cells in regeneration has also been observed in other systems including: anuran tadpole tails (Gargioli and Slack, 2004; Sugiura et al., 2004), chick lens (Park and Hollenberg, 1989), and mammalian liver (Fausto, 2000).

Therefore, we modified the nephrectomy protocol to remove most but not all proximal tubules. Since the nephrectomy protocol is manual, determining how much tissue is being removed is entirely subjective. However, after several iterations of this technique to determine the average percentage of tadpoles that can regenerate proximal tubules in three weeks, we typically find approximately 15-18% of nephrectomized



**Figure A1.3 Preliminary regeneration success study.** Complete nephrectomies correlate strongly with an absence of regenerated tubules at 21 dpn (63%). Conversely, while 18% of partial nephrectomies regenerate, much fewer are able to successfully regenerate after complete nephrectomies. “Partial” and “complete” assessments were made by eye.

tadpoles will regenerate a full kidney (Figure A1.3; Chapter 2 Figure 2.2, Caine and McLaughlin, 2013).

Still, a more objective method of removing proximal tubules would be a useful addition to these studies. For the preliminary study mentioned above (Figure A1.3), the amount of tissue removed was judged by eye. This is clearly a very imperfect method. Instead, we began sampling each batch of nephrectomized tadpoles at 1 dpn, to determine what percentage retained normal proximal tubule phenotype despite the surgery as well what percentage had partial tubule tissue, or an absence of tubules (Chapter 2, Figure 2.2, Caine and McLaughlin, 2013). This provided a relatively unbiased system for quantifying the outcome of each set of surgeries. While this type of data cannot indicate how much surviving tissue is needed for repair, it can demonstrate how many kidneys were not nephrectomized properly and thus should be subtracted from the 21 dpn regenerate group. It has also validated our original suspicions that complete nephrectomies correlate to a lack of regeneration (Chapter 2, Figure 2.2, compare white bars, Caine and McLaughlin, 2013).

Instead of sampling the population at 1 dpn, it would be more beneficial to be able to follow the same individual tadpoles from the nephrectomy through 21 dpn. To achieve this, we would need to develop a system to determine how much proximal tubule is being removed during the surgery without killing the animal. Our attempts to accomplish this have so far been unsuccessful. Excised pronephric tissue was not amenable to the harshness of our protein detection methods, thereby requiring alternative cell counting or tissue staining methods. It may be possible to visualize the pronephros via injected non-toxic dyes or fluorescent-macromolecules prior to nephrectomy, but this technique would

not be practically feasible as each individual would need to be injected, photographed, nephrectomized, then photographed again to be able to quantify how much labeled tissue was removed. This would also be difficult to do while minimizing exposure to anesthesia.

### *Stochasticity in regenerative results*

Unfortunately, even with all the aforementioned precautions, we occasionally obtain regeneration values that are difficult to explain. For example, we were unable to determine a specific reason for the unusually low percentage of regenerates seen at 21 dpn in the 5-7 dpn GM6001 (Chapter 4, Figure 4.1A) and SB-3CT (Chapter 4, Figure 4.2A) exposures. Other regenerative studies using ectothermic model organisms (fish and urodeles) have described similar problems, and attribute regenerative stochasticity to slight variations in temperature during an experiment (Poss et al., 2003) and the time of year that experiments are performed (Ghosh et al., 1994). In addition, differences in batches of tadpoles (fertilized on different days with different parents) can also contribute to variation in results (Slack et al., 2004). It is possible that these factors may have also been involved in the experimental outcomes observed in our system.

We also acknowledge that the percentage of nephrectomized tadpoles that were able to regenerate proximal tubules was much higher in the double GM6001 exposure study performed by the second experimenter (Chapter 4, Figure 4.3B) than in the preliminary study (Chapter 4, Figure 4.3A). This was especially apparent in the unexposed groups (18% compared to 41%; Figures 4.3A-B, black bars). These higher percentages, and the large standard error calculated from the unexposed treatment group, can be attributed to

one of the three replicates, which had an unusually high number of regenerates. This disparity is most likely due to a difference in nephrectomy technique between the two experimenters. Since the second experimenter had less experience with the technique, it is possible that less tubule was removed during these nephrectomies, thereby providing a greater advantage to this batch for successful regeneration. I saw similar results when I was first refining my technique (30% regeneration success). However, the variation in my technique has decreased considerably, and it appears that, in my hands, 15-18% of *Xenopus laevis* tadpoles are able to regenerate pronephric proximal tubules.

## **APPENDIX II**

### **Protocols and Recipes**



## PARTIAL UNILATERAL PRONEPHRECTOMY

### Reagents & Equipment

70% EtOH

2 pairs of fine (size 5) forceps

1% agarose dishes (made with 0.1X MMR)

Tricaine (5% stock)

0.1X MMR

Sterile medium and large Petri dishes for culturing tadpoles

### Protocol

1. Clean bench, microscope platform and 2 pairs of forceps with 70% EtOH.
2. Make up anesthetic: 0.04% tricaine in 0.1X MMR.
  - a. 120 $\mu$ l 5% tricaine in 15ml 0.1X MMR
3. Record your start time.
  - a. Keeping track of your start time for all surgeries will allow you to track your progress, speed, and improvements in your technique over time.
  - b. You will also need to know when you started if you are doing 0-1 dpn chemical exposures of any sort.
4. Anaesthetize 2 tadpoles for ~1 minute. Stage the tadpoles during this time. If they are too old or too young, euthanize and discard.
  - a. It helps to have a timer next to you counting up, so you can track the anesthesia time.
  - b. Use one tadpole from each pair as an unoperated control.
  - c. Nephrectomize the other tadpole.

5. To nephrectomize, pull left gill flap back with one pair of forceps (your dominant hand), while using the other pair to stabilize the tadpole.
  - a. Be careful not to puncture/squash the tadpole while stabilizing it.
  - b. If you cause any unintended damage, euthanize and discard the tadpole.  
If you nick the gut, remove too much kidney (e.g. if the duct is exposed),  
remove too much skin
6. Remove the proximal tubules (immediately below skin, in a little bundle).
7. Repeat 200 times.
8. Record your end time so that you can fix time-points to the hour.
  - a. You can also track your nephrectomy rate as your technique improves  
(number of tadpoles/hour).
9. Transfer each tadpole to fresh 0.1X MMR in a sterile Petri dish to recover from anesthesia at room temperature.
10. Rinse all tadpoles once with 0.1X MMR and transfer to fresh 0.1X MMR in a new, sterile Petri dish. Place dishes at 14°C overnight.
11. Record all data in a table (included).
  - a. Record number of edemas and dead tadpoles for first 3-5 days post-nephrectomy to assess health and mortality of the batch.
  - b. Make note of any abnormal or unique behavior, morphology or other observations you notice. Trust your instincts.

12. At 1 day post-nephrectomy (dpn):
  - a. Collect at least 20 tadpoles and fix in MEMFA at room temperature for 1 hour, then gradually dehydrate to 100% MeOH and store in -20°C until needed.
  - b. Change 0.1X MMR in remaining dishes and move tadpoles to 18°C.
13. Change 0.1X MMR daily & change dishes as necessary (you don't have to use sterile plates after 1 day post-nephrectomy).
  - a. Never stack more than 2 dishes at a time.
  - b. Alternate the top dish each day.
14. From NF stage 46 (~5 dpn), feed tadpoles daily with 0.008% green Sera<sup>®</sup> food in 0.1X MMR.
  - a. Make the food fresh daily.
15. Fix tadpoles at other time-points as necessary as in step 12.
16. **For calculating regeneration success for any batch, always fix some tadpoles at 1dpn and some at 21 dpn (as in step 12).**

### Table A2.1 Sample nephrectomy data collection sheet

[illegible]

## IMMUNOHISTOCHEMISTRY ON PARAFFIN-EMBEDDED SECTIONS

Modified from (Kelly and Melton, 2000)

### DAY 1

- 1.Circle section on back of slide with China marker
- 2.Thaw HI goat serum
- 3.Need 500ml 1X PBS
- 4.Need 8 Coplin jars
- 5.Need 2 humidified chambers (Tupperware with wet bench paper)
- 6.Make 250ml PTW (1X PBS, 0.1% Tween-20)

Remove 2.5ml from 250 ml 1X PBS

Add 2.5ml 10% Tween-20

- 7.Make 100ml **95 % EtOH** (if needed)

Add 95ml 100% EtOH to 5ml pH<sub>2</sub>O

- 8.Make 100ml **80 % EtOH** (if needed)

Add 80ml 100% EtOH to 20ml pH<sub>2</sub>O

- 9.Make 100ml **70 % EtOH** (if needed)

Add 70ml 100% EtOH to 30ml pH<sub>2</sub>O

10. Make 100ml **40 % EtOH** (if needed)

Add 40ml 100% EtOH to 60ml pH<sub>2</sub>O

11. Dewax/Deparaffinize

5' Xylene 1

5' Xylene 2

12. Rehydration

1' 100% EtOH 1

1' 100% EtOH 2

1' 95% EtOH

1' 80% EtOH

1' 70% EtOH

1' 40% EtOH

2' water

2' water

5' 1X PBS

2' 1X PBS

13. Make 50ml 0.3% H<sub>2</sub>O<sub>2</sub>

Add 500μl 30% H<sub>2</sub>O<sub>2</sub> to 49.5ml 1X PBS

14. 30' 0.3% H<sub>2</sub>O<sub>2</sub> to quench endogenous peroxidase activity

15. 10' PBS

16. 10' PBS

17. Make 500μl Block/slide

5% HI-goat serum/slide      25μl x \_\_\_\_ = \_\_\_\_

1% BSA/slide                  0.005g x \_\_\_\_ = \_\_\_\_

Up to 500μl PTW/slide      500μl x \_\_\_\_ = \_\_\_\_

18. 60' RT Block in humidified chamber

(record start time & end time in notebook)

19. O/N RT Primary Ab (use WIHC Ab) in humidified chamber (no coverslip necessary). (record concentration/date & start time in notebook)

**DAY 2 (Note start time in notebook)**

1. Need a humidified chamber (Tupperware with wet bench paper)
2. 5' PTW
3. 5' PTW
4. Make Secondary Ab (1:1500 in PTW as in WIHC → 600μl/slide)  

0.4μl 2°/slide	0.4μl x _____ = _____
600μl PTW/slide	600μl x _____ = _____
5. 60' RT Secondary Ab in humidified chamber (record start time & end time in notebook)
6. 5' PTW
7. 5' PTW
8. Make 150-200ml Chrom (same as WIHC, Levamisole optional)
9. 5' Chrom
10. 5' Chrom
11. Make 40ml BCIP/NBT in Chrom  

Add 133μl BCIP (1:300 – same as in WISH)
Add 13.3μl NBT (1:3000 – same as in WISH)
Up to 40ml Chrom
12. 30' Chrom/BCIP/NBT in Coplin jar wrapped in foil  
(record start time & end time in notebook)

13. 5' 1X PBS

14. 5' 1X PBS

15. 5' Running tap water

16. Counterstain or Coverslip (See protocol)



## ***IN SITU* HYBRIDIZATION ON PARAFFIN SECTIONS**

Modified from Hiroki Kuroda's protocol (attached)

### **20X SSPE**

175.3g NaCl  
27.6 g of NaH<sub>2</sub>PO<sub>4</sub>  
7.4 g of EDTA  
pH 7.4 (80+ pellets)  
Adjust volume to 1L with PicoH<sub>2</sub>O  
Add 10ml DEPC treat  
Autoclave

### **Kuroda's Hybridization Solution**

Make 1L  
Filter  
Store at -20°C in aliquots  
10g Boehringer Block  
500ml Formamide  
250ml 20X SSC  
Heat at 65 °C for 1 hour  
Add 120ml DEPC treated water  
100ml Torula RNA (10mg/ml in water; filtered)  
2ml Heparin (50mg/ml in 1X SSC)  
5ml 20% Tween-20  
10ml 10% CHAPS  
10ml 0.5M EDTA

### **Preparation**

1. Check availability & sign up for Hyb oven for O/N & Day 1
2. Check for 100ml DepC 1X PBS (Make if necessary)
3. O/N 37°C Dry slides
4. Wash 4 Coplin jars & 2 Incubation trays with DepC H<sub>2</sub>O (250ml)

Add 2.5ml DepC Stock (-20°C) to 250ml pH<sub>2</sub>O

## DAY 1

1. Thaw 20% PFA (8ml/Coplin jar) and Hyb at 65°C (500µl/slide)
2. Circle sections on back of slide with China marker
3. Make 40ml 70% EtOH (if needed)

Add 28ml 100% EtOH to 12ml pH<sub>2</sub>O

4. Make 500ml 2X SSPE

Remove 50ml DepC H<sub>2</sub>O

Add 50ml 20X SSPE

5. Make 40ml PTW (1X DepC PBS with 0.1% Tween-20)

\*Note: 100ml 1X DEPC PBS made → Pour 40ml in Conical tube for use in

Step 14

Remove 400µl 1X DepC PBS

Add 400µl 10% DepC Tween-20

6. Cool 20% PFA to RT
7. Dewaxing
  - 5' Xylene 1
  - 5' Xylene 2
8. Turn on Hyb oven to 37°C (if occupied, use big incubator by hood)
9. Rehydration
  - 1' 100% EtOH 1
  - 1' 100% EtOH 2
  - 1' 70% EtOH
  - 5' 2X SSPE (in Coplin jar)

10. Make 40ml 4% Paraformaldehyde

Add 8ml 20% PFA to 32ml DepC PBS

11. 15' RT Refix in 4% Paraformaldehyde/PBS (in Coplin jar)

12. 5' 2X SSPE & Thaw PK at RT

13. 5' 2X SSPE

14. Make 40ml 3μg/ml Proteinase K

60μl 2mg/ml PK in 40ml PTW

15. 30' 37°C 3μg/ml PK (in Coplin jar)

(Note: This step is for making RNA more accessible to hybridization)

16. Make 40ml 0.2M HCl

Add 8ml 1M HCl (hood) to 32ml DepC H<sub>2</sub>O

17. Turn up Hyb oven to 65°C

18. 5' 2X SSPE

19. 5' 2X SSPE

20. 15' RT 0.2M HCl (in Coplin jar)

(Note: This step is to denature proteins, nick DNA, and partially reverse the fix)

21. 5' 2X SSPE

22. 5' 2X SSPE

23. 2 hours 65°C Add 500μl of hybridization buffer to each slide and incubate in a

humidified chamber (record actual time length in notebook)

24. Warm probes (need 100μl per slide)

25. DEPC-treat coverslips (100ml pH<sub>2</sub>O + 1ml DEPC Stock)

26. Drain off excess hybridization buffer

27. Add 110µl probe solution to each slide (record which probe used in notebook)
28. Cover with DEPC glass coverslips ON A LEVEL SURFACE (or coverslips will slide off!)
29. O/N 65°C Incubate slides in a humidified chamber (record actual time length in notebook)

**DAY 2** (Note start time in notebook)

1. Thaw Hyb at 60°C (~800µl/slide)                      800µl x \_\_\_\_\_ = \_\_\_\_\_
2. Turn incubator by hood down to 4°C
3. Soak slides in 40ml 2X SSPE until the coverslips fall off (in Coplin jar)
4. Make 50% Hyb/50% 2X SSPE/0.3% CHAPS (Need at least 500µl per slide)

300µl/slide Hyb	300µl x number of slides = _____
300µl/slide 2X SSPE	300µl x number of slides = _____
0.002g/slide CHAPS (4°C door)	0.0015g x number of slides = _____

Vortex & leave at RT
5. Cool Hyb to RT
6. Thaw HI Serum at RT
7. Turn water bath up to 70°C
8. Turn Hyb oven back to 60°C and turn off
9. Make Antibody (Ab) Buffer (Need 750µl/side)

Add 75µl/slide 100% HI Goat Serum → 10% (final concentration)  
Add 0.0075g/slide Boehringer Blocking Reagent (Roche #10961776) → 1%  
Add 7.5µl/slide 10% Tween-20 → 0.1%  
Up to 750µl/slide 1X PBS  
**Dissolve at 70°C & Vortex frequently**

10. 5' RT Cover slide with 500 $\mu$ l hybridization buffer
11. Drain slide
12. 10' RT Add 500 $\mu$ l 50 % Hyb: 50% 2X SSPE/0.3% CHAPS
13. Make 500ml PTW  $\rightarrow$  Remove 5ml 1X PBS & Add 5ml 10% Tween-20 (Not DepC)
14. 20' Soak in 2X SSPE
15. Vortex Ab Buffer
16. 10' PTW & Vortex Ab Buffer (in Coplin jar)
17. 10' PTW & Vortex Ab Buffer
18. 10' PTW & Vortex Ab Buffer
19. Turn 70°C water bath back down to 60°C and turn off
20. Filter Ab Buffer with 0.45 $\mu$ m filter into Conical tube
- 21. Add  $\alpha$ -dig-AP 2° Ab to Antibody Buffer** (Diluted 1:1000  $\rightarrow$  0.2 $\mu$ l:200 $\mu$ l per slide)
22. 2 hours RT Add 500  $\mu$ l of Antibody Buffer to each slide in a humidified chamber  
(record actual time length in notebook)
23. 2 hours rocking 4°C, Pre-block the Antibody in Antibody Buffer  
(record actual time length in notebook)
24. Make 150ml 0.1% BSA in PTW  
0.15g BSA in 150ml PTW
25. Drain slides
26. 60' RT Cover slide with 200 $\mu$ l of Pre-blocked Antibody (record actual time length in notebook)
27. 10' 0.1 % BSA in PTW (in Coplin jar)
28. 10' 0.1 % BSA in PTW

29. 10' 0.1 % BSA in PTW

30. Make 200ml Alkaline Phosphatase (AP) Buffer

Add 20ml 1M Tris, pH 9.5 → 0.1M (final concentration)

Add 4ml 5M NaCl → 0.1M

Add 10ml 1M MgCl<sub>2</sub> → 0.05M

Up to 200ml dH<sub>2</sub>O

31. 5' Rinse slides in AP buffer (in Coplin jar)

32. 5' Rinse slides in AP buffer (in Coplin jar)

33. Make BCIP/NBT in AP Buffer

Add 133.3μl BCIP → 1:300 (same as in WISH)

Add 13.33μl NBT → 1:3000 (same as in WISH)

Up to 40ml AP Buffer

34. Detection of expression:

4°C (Dark) Incubate in BCIP/NBT in AP Buffer (in Coplin jar)

Cover in foil!      Record time for expression to appear

35. Make 50ml Stop Solution (pH 7.4)

Add 5ml 1M Tris pH 7.4 → 100mM pH 7.4

Add 100μl 0.5M EDTA pH 8.0 → 1mM

Up to 50ml dH<sub>2</sub>O

36. 5' PTW

37. 5' PTW

38. 15' Stop the reaction with Stop Solution (in Coplin jar)

39. Counterstain or Coverslip (See protocol)

**Table A2.2 Kuroda SISH Protocol.**

<p><b>In situ hybridization on Sections - Hiroki Kuroda (1st day)</b> Note. Embed sample in paraffin in HE staining case. 1. Cut samples at 20-25 µm thickness 2. Dry slides for overnight at 45°C. <b>(2nd day)</b> 1. Rehydrate (Xylene for 5 min) x2 (100% EtOH for 1 min) x2 70% EtOH for 1 min 2 x SSPE 2. Refix in 4% Paraformaldehyde in PBS at RT for 15 min. 3. Rinse with 2 x SSPE 4. Incubate slides in Proteinase K (3 µg/ml in PBSw) at 37°C for 30 min. Note: This step is for making RNA more accessible to hybridization. 5. Rinse slides in 2x SSPE 6. Incubate slides in 0.2M HCl at room temperature for 15 min. Note: This step is to denature proteins, nick DNA, and partially reverse the fixation step. 7. Rinse with 2x SSPE. 8. Add 400 µl of hybridization buffer to each slide and incubate in a humid chamber at 65 °C for 2 hours. 9. Drain off excess hybridization buffer. 10. Add 110 µl of 0.5 µg/ml probe solution to each slide. 11. Put glass coverslips on the top. 12. Incubate slides in a humid chamber at 65°C overnight. <b>(3rd day)</b> 1. Soak slides in 2x SSPE until the coverslips fall off (in a Coplin jar). 2. Add enough hybridization buffer to cover the slide and incubate at room temperature for 5 min. 3. Drain slides and add 50 % hybridization buffer: 50% 2x SSPE: 0.3% CHAPS. 4. Incubate at RT for 10 min. 5. Soak in 2x SSPE for 20 minutes (in a Coplin jar). 6. (Rinse slides in PBSw for 10 min in a Coplin jar) x3 7. Add 500 µl of Antibody Buffer to each slide. 8. Incubate for 2 h at RT. Note: At the same time, pre-block the antibody (anti-dig AP fab fragments, diluted :1000) in the Antibody Buffer at 4°C, rocking for 2 hours. 9. Drain slides and add 200 µl of pre-blocked antibody and incubate at RT for 1 h. 10. (Rinse slides in 0.1 % BSA in PBSw for 10 min in a Coplin jar) x3. 11. Rinse slides in AP buffer for 10 min in a Coplin jar. 12. Begin staining by 10-fold diluted BM purple with AP buffer and incubate at 4°C in the dark until the strength of staining is appropriate. 13. Wash in PBSw and then stop the reaction with Stop Solution for 15 min.</p>	<p><b>Solutions:</b> <b>10x PBS:</b> 80g NaCl, 2g KCl, 14.4g Na<sub>2</sub>HPO<sub>4</sub>, 2.4g KH<sub>2</sub>PO<sub>4</sub>, pH to 7.4,. Adjust volume to 1L with DDW, DEPC treat and autoclave. <b>PBSw:</b> PBS with 0.1% Tween-20 <b>20x SSPE:</b> 175.3g NaCl, 27.6 g of NaH<sub>2</sub>PO<sub>4</sub>, 7.4 g of EDTA, pH 7.4, adjust volume to 1L with DDW, DEPC treat and autoclave. <b>Hybridization Solution:</b> Make 1L, filter, and store at 20°C in aliquots.(1st step) 10g Boehringer Block, 500ml Formamide, 250ml 20x SSC, Heat at 65 °C for 1 hour. Add 120ml DEPC treated water, 100ml Torula RNA (10mg/ml in water; filtered), 2ml Heparin (50mg/ml in 1x SSC), 5ml 20% Tween-20, 10ml 10% CHAPS, 10ml 0.5M EDTA. <b>Antibody Buffer:</b> 10% Heat Inactivated Goat Serum, 1% Boehringer Block, 0.1% Tween-20 Dissolve in PBS at 70 °C, vortexing frequently, and then filter (0.45 µm). <b>AP buffer:</b> Put 5 ml of 1M NaCl, 1M Tris, pH 9.5 and 5 ml of 0.5M MgCl<sub>2</sub> into 40 ml of DDW. Note. Do not mix at high concentration, or precipitate will appear. <b>Stop Solution:</b> 100mM Tris pH7.4, 1mM EDTA <b>Product:</b> Boehringer Block - Roche #1096176 Proteinase K - Gibco #25530-049 Anti-Dig-AP - Roche #1093274 BM purple – Roche</p> <p>Source:  <a href="http://www.hhmi.ucla.edu/derobertis/protocol_page/Pdfs/Frog%20protocols/In%20situ%20hybridization%20on%20Sections.pdf">http://www.hhmi.ucla.edu/derobertis/protocol_page/Pdfs/Frog%20protocols/In%20situ%20hybridization%20on%20Sections.pdf</a></p>
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## COUNTERSTAINING PARAFFIN SECTIONS AFTER CHEMISTRIES

Make 50ml 1% Eosin (if necessary) → Pink cytoplasmic stain

For stock: 0.5g in 50ml 70% EtOH.....To Coplin Jar: Add 1 drop of glacial acetic acid

Make 50ml 20% Hematoxylin (if necessary) → Blue nuclear stain

10ml Harris' Hematoxylin (in flammables cabinet)

40ml dH<sub>2</sub>O

1. 3' Xylene 1
2. 1' Xylene 2
3. 1' Xylene 3
4. 1' 100% EtOH
5. 1' 95% EtOH
6. 1' 70% EtOH
7. 5' Tap H<sub>2</sub>O

### **8. Optional:**

- a. 90 sec 20% Harris' Hematoxylin
- b. 5' Running tap water
9. 5' 1% Eosin in 70% EtOH
10. 5' Running tap water
11. 1' 70% EtOH
12. 1' 95% EtOH
13. 1' 100% EtOH
40. 1' Xylene 1
41. 5' Xylene 2
42. Mount with 2-3 drops Permount and coverslip (on foil tray)
43. Let dry O/N in fume hood



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