Characterization of TGFβ signaling during epimorphic tissue regeneration: an example using the leopard gecko (*Eublepharis macularius*) tail regeneration model.

by

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ABSTRACT

Characterization of TGFβ signaling during epimorphic tissue regeneration. An example using the Leopard Gecko (*Eublepharis macularius*) tail regeneration model.

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The transforming growth factor beta (TGFβ)/activin signaling pathway has a number of documented roles during wound healing and is becoming increasingly appreciated as a vital component of multi-tissue regeneration. The leopard gecko (*Eublepharis macularius*) is able to spontaneously, and repeatedly, regenerate its tail following tail loss. We thus examined the expression and localization of several key components of the TGFβ/activin signaling pathway during tail regeneration of the leopard gecko. We observed a marked increase in phosphorylated-Smad2 expression among regenerating tissues corresponding to the location of the regenerate blastema. Interestingly, we observe that during early regeneration there appears to be an absence of TGFβ family member TGFβ1 and instead a strong upregulation of activin-βA. We also observe the expression of EMT transcription factors Snail1 and Snail2 in blastemal tissue. These observations combined with other data provide strong support for the importance of unique and non-overlapping expression patterns of different TGFβ ligands during multi-tissue regeneration.
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Declaration of Work Performed

I declare that with the exception of the items indicated below, all work reported in this thesis was performed by me.

Members of the Vickaryous lab prepared all of the slides used for immunostaining (embedding and sectioning) staining was performed by Richard W.D. Gilbert.
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List of Abbreviations
ALK     Activin-like Kinase
APS     Ammonium Persulfate
BMP     Bone Morphogenic Protein
BSA     Bovine Serum Albumin
DAPI    4’, 6’-Diamidino-2phenylindole
DTT     Dithiothreitol
ECM     Extracellular Matrix
EMT     Epithelial to Mesenchymal Transition
GDF     Growth and Differentiation Factor
HRP     Horseradish Peroxidase
IF      Immunofluorescence
IHC     Immunohistochemistry
LAP     Latency Associated Peptide
LLC     Large Latency Complex
LTBP    Latent TGFβ Binding Protein
MRL     Murphy Roths Large
PCR     Polymerase Chain Reaction
PBS     Phosphate-buffered Saline
PMSF    Phenylmethylsulfonyl Fluoride
PVDF    Polyvinylidene Fluoride
RCF     Relative Centrifugal Force
SDS     Sodium Dodecyl Sulfate
SLC     Small Latency Complex
SMAD    Small Body and Mothers Against Decapentaplegic Homolog
pSMAD   Phosphorylated SMAD
TβR     Transforming Growth Factor β Receptor
TBS     Tris-buffered Saline
TGFβ    Transforming Growth Factor Beta
TEMED   Tetramethylethylenediamine
Introduction

Among amniotes, the most common response to traumatic injury is the rapid formation of fibrotic tissue. Although fibrotic (or scar) tissue is often capable of stabilizing the injury and re-establishing tissue homeostasis, it is a dysfunctional and structurally dissimilar substitute (Martin, 1997; Ferguson and O’Kane, 2004; Gurtner et al., 2008). In contrast, some amniotes, along with various urodeles (newts, axolotls) and teleosts (zebrafish), are capable of scar-free wound healing (Tanaka and Reddien, 2011). Significantly, scar-free wound healing is generally followed by tissue regeneration and, in many instances, the restoration of function. To date, the best-known examples of scar-free wound healing and regeneration in amniotes include: early embryos, (Whitby and Ferguson, 1991; reviewed by Ferguson and O’Kane, 2004); the digit tips of neonate and adult mice, (Han et al., 2008; Fernando et al., 2011; Rinkevich et al., 2011); and the tail of many lizard species (Bellairs and Bryant, 1985; Alibardi, 2010; Delorme et al., 2012). Lizards in particular provide an interesting example in that scar-free wound healing and regeneration is typically associated with caudal autotomy, an anti-predation strategy whereby the tail is self-detached. Tail loss yields a large cross-sectional wound, with the spinal cord, skeletal muscle, dermis and tail vertebrae all exposed (Bellairs and Bryant, 1985; McLean and Vickaryous, 2011). Spontaneously, this wound is rapidly sealed without the formation of fibrotic tissue, and a replacement tail is soon developed (Alibardi, 2010; McLean and Vickaryous, 2011; Delorme et al., 2012). The replacement tail arises from a blastema, an accumulation of highly proliferative, mesenchymal-like cells that rapidly populate the site of tail loss.
Whereas the endogenous source of blastemal cells in lizards remains uncertain, evidence from the study of various other scar-free wound healing and regenerating species, including urodeles (Kragl et al., 2009) and mice (digit tips: Rinkevich et al., 2011) strongly supports dedifferentiation of lineage-restricted progenitor cells and not pluripotent stem populations.

An emerging model for the study of scar-free wound healing and regeneration in lizards is the leopard gecko, *Eublepharis macularius* (Whimster, 1978; McLean and Vickaryous, 2011; Delorme et al., 2012). Previous work in the leopard gecko has established that wound healing and regeneration are highly conserved processes and that the entire regenerative process can be divided into seven discrete morphological stages (McLean and Vickaryous, 2011). Stages I to III primarily involve wound healing, including wound site contraction, the formation of an exudate clot, re-epithelialization of the wound site, and the initiation of blastema formation. Stages IV to VII involve tissue outgrowth and differentiation, including angiogenesis, axonogenesis, spinal cord outgrowth, and muscle and skeletal development (McLean and Vickaryous, 2011). Although the molecular regulation of these events remains poorly understood, evidence from various other regeneration-competent models supports the involvement of various cytokines (Stoick-Cooper et al., 2007; Stocum and Cameron, 2011).

The common canonical signaling pathway shared by transforming growth factor beta (TGFβ) and activin ligands, referred to as the TGFβ/activin pathway (Ogunjimi
et al., 2012), has been shown to be essential for the regenerative response in other models of multi-tissue regeneration (Jazwinska et al., 2007; Levesque et al., 2007; Ho and Whitman, 2008). TGFβ and activin are members of the TGFβ superfamily of ligands which signal through specific sets of serine/threonine kinase receptors (Moustakas and Heldin, 2009). Upon ligand binding, TGFβ/activin receptors become activated and subsequently recruit and phosphorylate specific signaling intermediates known as receptor regulated SMADs (R-SMADs) (Wu and Hill, 2009). TGFβ and activin cause the phosphorylation of a particular subset of R-SMADs, SMAD2 and SMAD3 (Ross and Hill, 2008). These phosphorylated SMADs then form a complex with a common mediator, SMAD4 and subsequently translocate to the nucleus and drive a variety of gene expression programs (Ross and Hill, 2008). The previous studies examining TGFβ/activin signaling in regeneration have shown that when the kinase domains of the TGFβ and activin receptors are pan-inhibited in the regenerating appendage through the use of small molecule inhibitors, multi-tissue regeneration is blocked (Inman et al., 2002; DaCosta Byfield et al., 2004). Additionally, when the activin arm of the TGFβ/activin signaling pathway was silenced in regenerating zebrafish tail fins using knockdown morpholinos, regeneration was partially blocked (50-80% reduction in regeneration) (Jazwinska et al., 2007). Taken together, these results suggest that various TGFβ/activin ligands (such as TGFβ1-3, and/or activin βA/ββ) are playing vital, ligand specific roles in the multi tissue regenerative process.

Despite these previous studies, the role of TGFβ/activin signaling during multi-
tissue regeneration remains poorly understood. In particular, the specific expression patterns of various TGFβ/activin ligands have not been extensively characterized and it is likely that different ligands are playing differing roles at different times throughout regeneration. For example, previous work using the leopard gecko observed exclusive TGFβ3 expression in chondrocytes and fibroblasts only at the later stages of regeneration (VI and VII) (Delorme et al., 2012). These observations, combined with those of previous studies in zebrafish (Jazwinska et al., 2007), axolotl (Levesque et al., 2007) and Xenopus (Ho and Whitman, 2008) suggesting variable TGFβ ligand expression throughout regeneration suggests that differential and dynamic expression of individual TGFβ/activin ligands mediate unique cellular events during regeneration.

With this in mind we sought to further investigate the TGFβ/activin signaling pathway at multiple stages during the leopard gecko regenerative program (from stages III to VI). In particular, we focused on the pro-fibrotic cytokine TGFβ1 which has been associated with scar formation and fibrosis in numerous physiological and pathological situations (Bielefeld et al., 2012). We also set out to identifying novel, TGFβ/activin mediated transcriptional targets that could be contributing to the regenerative phenotype seen in lizards. In particular, we focused on well-documented mediators of the TGFβ-induced epithelial to mesenchymal transition (EMT), a phenomenon of cellular plasticity involved in the reactivation of stem cell features in epithelial cells that has yet to be examined in the context of multi-tissue regeneration (Lim and Thiery, 2012)
Review of Literature

Transforming Growth Factor Beta (TGFβ) Super-family:

In humans, the TGFβ family consists of 33 members, most of which are dimeric, secreted polypeptides (Moustakas and Heldin, 2009). In addition to the three prototypic TGFβ protein isoforms (TGFβ1, TGFβ2 and TGFβ3), the family also includes the activins, bone morphogenic proteins (BMPs), growth and differentiation factors (GDFs), myostatin and nodal (reviewed by Derynck and Miyazono, 2008). The TGFβ family is conserved throughout metazoan evolution, with ligands being found in both vertebrates and invertebrates (Hinck, 2012; Oshimori and Fuchs, 2012). At the cellular level, TGFβ family members regulate growth, differentiation, adhesion, migration and death in a context-dependent, dose-dependent and cell type specific manner (Moustakas and Heldin, 2009). TGFβ family members have also been implicated in a variety of physiological and pathological processes including development (reviewed by Moustakas and Heldin, 2009), stem cell maintenance (reviewed by Beyer et al., 2012; Oshimori and Fuchs, 2012), fibrosis, autoimmune disease, cardiovascular disease (reviewed by Gordon and Blobe, 2008), and cancer (reviewed by Massague, 2008). In addition, ligands from the TGFβ superfamily have documented roles during wound healing and regeneration (Jazwinska et al., 2007; Levesque et al., 2007; Ho and Whitman, 2008; Bielefeld et al., 2012).
**TGFβ Ligands:**

Among mammals, the TGFβ isoforms TGFβ1, TGFβ2 and TGFβ3 are multifunctional cytokines that act in autocrine and paracrine manners to regulate a diverse array of cellular processes (ten Dijke and Arthur, 2007). Each of these isoforms is encoded by a separate gene, with the preprotein sequence consisting of a signal peptide (~30 kDa) and the “mature” TGFβ sequence (~12.5 kDa) (Munger and Sheppard, 2011). After proteolytic processing in the endoplasmic reticulum, the TGFβ monomers form homodimers through disulfide bonding, resulting in the formation of the precursor molecule, pro-TGFβ (ten Dijke and Arthur, 2007). The pro-TGFβ dimer is then further processed (by furin convertase) to yield a small latent complex (SLC) consisting of the mature TGFβ protein and a latency associate peptide (LAP) (Dubois et al., 1995). The SLC (mature TGFβ protein plus LAP) then forms a complex with the large latent TGFβ binding protein (LTBP) to yield the large latency complex (LLC) (Rifkin, 2005). Once secreted the LLC interacts through its N-terminal with various extracellular matrix components to allow TGFβ to become deposited in the ECM (ten Dijke and Arthur, 2007)

**TGFβ Activation**

In order for latent TGFβ to become activated, the mature TGFβ dimer must be released from the LLC (TGFβ-LAP-LTBP) complex embedded in the ECM. This can be achieved through a variety of different mechanisms in various cellular contexts, all
of which directly target the LAP (reviewed by ten Dijke and Arthur, 2007). For example, in vivo it has been demonstrated that binding of secreted thrombospondin-1 to the LAP disrupts the non-covalent bonds holding TGFβ, leading to release and activation (Crawford et al., 1998). Furthermore, it is known that latent TGFβ can be activated by αvβ6 and αvβ8 integrins, which bind the RGD sequence in the LAP and cause a conformational change (Sheppard, 2005). The importance of TGFβ activation is demonstrated in mice with engineered mutations in various TGFβ activators exhibiting phenotypic recapitulations of mice deficient in TGFβ signaling components (Yang et al., 2007; summarized by ten Dijke and Arthur, 2007). As an example, it has been demonstrated that mice with an engineered mutation in the RGD sequence of the LAP recapitulate the main features of TGFβ1 null mice (Yang et al., 2007).

*TGFβ Ligand-Receptor Interactions:*

Once mature TGFβ peptides are released from the LAP, these ligands evoke their cellular effects in target cells by binding to transmembrane, dual specificity receptors (strong serine/threonine activity and weak tyrosine kinase activity)(Moustakas and Heldin, 2009). TGFβ receptors can be divided into three classes: type I, type II and type III (co-receptor) (TβRI, TβRII and TβRIII, respectively). Seven type I, five type II and two type III receptors have been identified in humans (Shi and Massague, 2003, Wrana, 2008) Type I and type II receptors have short extracellular cysteine-rich domains that are subject to
glycosylation, a single pass transmembrane domain, and an intracellular domain consisting mainly of the kinase catalytic motif (Wrana et al., 2008; Kang et al., 2009). TGFβ ligands (TGFβ 1-3) primarily bind to TβRII and TβRI, also known as activin receptor-like kinase 5 (ALK-5). To activate cell signaling, the TGFβ ligand first binds to the constitutively active TβRII, which is then brought into close proximity with TβRI (Wrana et al, 1994). This close interaction allows TβRII to phosphorylate the short GS domain (TTSGSGSG) located at the juxtamembrane region of TβRI.

Phosphorylation results in a conformational change to TβRI, leading to receptor activation (Souchelnytskyi et al., 1996; Kang et al., 2009). Evidence suggests that both TβRI and TβRII exist as homodimers on the cell membrane (Type I-Type I, Type II-Type II) but complex into heterotetramers in the presence of a ligand (Yamashita et al., 1994). As currently understood, all TGFβ signaling requires both TβRI and TβRII (Wrana et al., 2008). In support of this, it has been shown than TGFβ ligands only bind to TβRII with high affinity, thus making receptor heterotetramer complex formation critical for TβRI activation (Wrana et al., 2008). Once activated, the receptor complex initiates an intracellular cascade that evokes the activation of canonical and non-canonical signaling pathways (discussed below; reviewed by Moustakas and Heldin, 2005, 2009). Adding to the complexity of this process, some ligand-receptor interactions require a TβRIII co-receptor. For example, TβRII is unable to bind TGFβ2 without a co-receptor, such as betaglycan (De Crescenzo et al., 2006).
**Activin Ligands**

The TGFβ superfamily also includes activins, and the structurally and evolutionarily related inhibins that, similar to TGFβ ligands, are known to have diverse functions throughout the body, in particular during embryonic development and wound healing (Wiater and Vale, 2008). Activins and inhibins are both disulfide linked dimers consisting of various inhibin-α and inhibin-β polypeptide subunits. In humans there is a single inhibin-α polypeptide and four inhibin β polypeptides (βA, βB, βC, βD) all encoded by different genes (Vitt et al., 2001). The mature inhibin dimers (inhibin- A, B) exist as heterodimers of an inhibin-α subunit combined with an inhibin-β subunit (βA or βB) (Wiater and Vale, 2008). The mature activin dimers (Activin A, B, AB) exist as homodimers of various inhibin β subunits (Burger et al., 1988; Vale et al., 1988). Similar to TGFβ, activin and inhibin subunits are initially synthesized as large precursor proteins that require proteolytic processing for proper folding and secretion. Briefly, the prodomains of the inhibin subunits are cleaved after dimer assembly to yield the mature activin or inhibin polypeptide (Werner and Vale, 2008). Unlike TGFβ ligands, the pro-domain of activin and inhibin does not stay in contact with the mature peptide and has no apparent biological effect (Wiater and Vale, 2008).

**Activin Receptor Binding**

As for TGFβ ligands, activins induce responses in target cells by binding to type II
and type I receptors. Mammals have two activin type II receptors, ActRIIA and ActRIIB, and one activin type I receptor, ActRIB (also known as Alk-4) (Carcamo et al., 1994; Attisano et al., 1996). The molecular interactions between the various activin ligands and their receptors are similar to those of TGFβ ligands. For example, similar to the TGFβ ligand receptor interactions, activin interacts first with ActRIIA or B and then ActRIB, causing the activation of the type I kinase domains (Wiater and Vale, 2008). It is suggested that activin-mediated activation involves the same canonical and non-canonical pathways observed following activation with TGFβ ligands, however this is likely an oversimplification and down stream signaling differences are highly probable (Moustakas and Heldin, 2005; Wiater and Vale, 2008; Moustakas and Heldin, 2009).

Inhibin ligands are known to antagonize the actions of activins particularly among tissues of the reproductive axis (Harrison et al., 2005). Inhibin antagonism of activin signaling results from competition for ActRIIA or B, mediated through receptor binding to the shared inhibin β subunit (βA or βB) (Gray et al., 2000). It is worth noting however, that inhibin affinity for ActRIIB is ten-times lower than activin, and that in some tissues inhibin cannot readily antagonize activin (Lewis et al., 2000). It has been demonstrated that the type III TGFβ co-receptor betaglycan can act as a co-receptor for inhibin, leading to a thirty-fold increase in inhibin binding affinity at the ActRIIB (Lewis et al., 2000). Interestingly, betaglycan is differentially expressed in tissues throughout the body, and in the rat, betaglycan mRNA is expressed in the brain, pituitary gland and gonads, pointing to tissue specific roles for inhibin
(MacConell et al., 2002).

Canonical Signalling

The canonical TGFβ/activin signaling pathway is mediated through cytoplasmic proteins known as SMADs (Small body and mothers against decapentaplegic homolog) (reviewed by Feng and Derynck, 2005). In vertebrates there are eight members of the SMAD family, SMADs 1-8. Each SMAD protein consists of three domains: (1) an N-terminal Mad-homology (MH) 1 domain that can interact with other proteins and contains the nuclear localization signal as well as DNA binding elements; (2) a middle linker domain that interacts with ubiquitin ligases; and (3) a C-terminal MH2 domain that binds type I receptors, interacts with other proteins, and mediates SMAD homo- and hetero-oligomerization (Feng and Derynck, 2005; Moustakas and Heldin, 2009). SMADs are further categorized into three classes depending on their structure and function. Receptor activated or R-SMADs (SMADS 1-3,5,8) interact with activated type I receptors resulting in phosphorylation of their conserved SSXS motif, located at the protein carboxy-terminus (C-terminus) (Feng and Derynck, 2005). Upon C-terminal phosphorylation the common-mediator SMAD, Co-SMAD (SMAD4), interacts with activated R-SMAD complexes to assist their nuclear translocation. Inhibitory or I-SMADs (SMADs 6,7) interact with activated type I receptors and inactivate the receptor complex with the help of the E3 ubiquitin ligase Smurf 1/2 (Feng and Derynck, 2005; Moustakas and Heldin, 2009).
The five known R-SMADs (SMADs 1-3,5,8) are phosphorylated in response to different TGFβ family ligands that bind various TGFβ receptor combinations. In most cases TGFβ, activin, myostatin and nodal ligands cause C-terminal phosphorylation of SMAD2 and SMAD3, whereas BMPs and GDFs cause the C-terminal phosphorylation of SMAD1, SMAD5 and SMAD8 (Ross and Hill, 2007). However, it has been reported that TGFβ1-3 can cause C-terminal phosphorylation of SMAD1/5/8 through a cell-type specific type I receptor (ALK-1) found on endothelial cells (Goumans et al., 2002). Upon receptor-induced C-terminal phosphorylation, R-SMADs interact with SMAD4 (the co-SMAD), forming activated SMAD complexes. SMAD4 is essential for most SMAD transcriptional complex formation as well as nuclear translocation, and is not ligand restricted (i.e., SMAD4 can form complexes with all R-SMADS). In response to TGFβ/activin signaling, activated SMAD complexes may form heterodimers (SMAD2 or 3-SMAD4) or heterotrimers (SMAD2-SMAD3-SMAD4), depending on the target gene. These complexes accumulate in the nucleus and can regulate gene expression both positively and negatively often in association with other transcription factors (Feng and Derynck, 2005; Ross and Hill, 2007). Adding to the complexity of SMAD signalling, each of the R-SMAD proteins contains numerous sites for post-translational modification, including multiple phosphorylation sites, sumolation sites, as well as acetylation sites. These modifications can cause numerous effects on SMAD signalling, further reviewed by (Moustakas and Heldin, 2009, Xu et al., 2012)
Non-Canonical Signaling

Whereas TGFβ/activin ligands primarily signal through the canonical SMAD pathway, numerous non-canonical/non-SMAD signaling pathways have also been demonstrated to be activated by TGFβ ligands. These include the Ras/MAPK/Erk pathway, the PI3K/Akt pathway, the JNK/SAPK pathway, the p38 MAPK pathway, and the Par6-Polarity pathway. Non-canonical pathways play diverse roles in modulating SMAD mediated and non-SMAD mediated signaling events, and can be activated directly or indirectly by the TGFβ receptor complex. The importance of non-canonical signaling in determining the functional outcome of TGFβ/activin signaling has been previously demonstrated (Ozdamar et al., 2005; Lee et al., 2007; Sorrentino et al., 2008; Yamashita et al., 2008). This thesis will focus on canonical signaling, given its well-documented role in tissue regeneration (Jazwinska et al., 2007; Levesque et al., 2007; Ho and Whitman, 2008). A recent review (Zhang et al., 2009) presents an extensive discussion on non-canonical signaling and its role in TGFβ’s multiple cellular effects.

TGFβ isoforms in physiology and pathology.

Despite sharing 71-76% sequence identity and signaling through the same canonical SMAD intermediates, a growing body of evidence suggests that the three TGFβ isoforms as well as the various activin isoforms cause different transcriptional effects (Ferguson and O’Kane, 2004, Nawshad et al., 2004). In particular, it has been
observed that TGFβ 1/2/3 and activin knockout mice all demonstrate distinct, generally non-overlapping phenotypes.

*TGFβ and activin knockout animals*

Targeted disruption of *TGFβ1* gene leads to hematopoetic and vasculogenic defects that result in the death of nearly 50% of null embryos by 10 days of gestation (Dickson et al., 1995). The remaining embryos eventually die of wasting syndrome and multi-organ failure as a result of excessive multifocal inflammatory disease (Shull et al., 1992). TGFβ2 knockout mice exhibit perinatal mortality as a result of multiple developmental abnormalities affecting the cardiopulmonary, urogenital, visual, auditory, neural and skeletal systems (Sanford et al., 1997). Mice lacking TGFβ3 exhibit cleft palate and die immediately after birth due to an inability to suckle effectively (Proetzel et al., 1995; Kaartinen et al., 1995; Laverty et al., 2009). Due to overlap between activin and inhibin (mRNA transcripts that encode activin monomers also make up components of the inhibin system), accurate interpretation of activin knockout mice remains problematic (Vale and Werner, 2008). It is worth noting however, that *activin-βA* knockout mice demonstrate delayed vibrissae follicle formation and (similar to *TGFβ3* knockouts) cleft palate abnormalities (Matzuk et al., 1995; McDowall et al., 2008).

*SMAD isoform specific effects*
Similar to the various isoforms of TGFβ and activin, SMAD2 and SMAD3 appear to have different roles during development as well. SMAD2 and 3 are highly conserved at the protein level, with 83.9% homology (Petersen et al., 2010). However, there is an important structural difference in the MH1 domain, where SMAD2 contains two short peptide inserts that impose steric constraints that prevent it from binding DNA, whereas SMAD3 retains DNA binding capacity (Dennler et al., 1999). In addition to structural differences, mice deficient in SMAD2 are embryonic lethal whereas SMAD3 deficient mice are viable (Weinstein et al., 1998; Ashcroft et al., 1999; Yang et al., 1999). Interestingly, keratinocyte specific SMAD3 knockout yields mice that are resistant to carcinogen induced skin cancer while keratinocyte specific SMAD2 knockout leads to accelerated formation and malignant progression of carcinogen induced tumors suggesting distinct transcriptional effects (Hoot et al., 2008).

**Cutaneous Wound Healing and TGFβ/activin isoforms**

One of the best examples highlighting the complexity of the various TGFβ/activin isoforms in the determination of physiological and pathological outcomes is cutaneous wound healing. Cutaneous wound healing is a highly dynamic process regulated through numerous cellular and molecular interactions (Rienke and Sorg, 2012). In adult mammals cutaneous wound repair involves successives stages of hemostasis, inflammation, granulation, and subsequent reepithelization resulting in the formation of scar tissue (Rienke and Sorg, 2012). Mature mammalian scar tissue
is characterized as avascular and acellular consisting of mainly disorganized ECM (Reinke and Song, 2012). Compared to post-natal mammalian wound healing, mammalian fetuses retain the remarkable ability to undergo scar-free wound healing (Rowlatt, 1979; Ferguson and O’Kane, 1996; Colwell et al., 2006). In response to numerous tissue injuries, fetal dermis and epidermis regenerates nondisrupted ECM and dermal appendages indistinguishable from original tissue (Coolen et al., 2010). This scar-free wound healing capacity has been attributed to numerous factors including ECM differences, altered inflammatory response, differential gene/protein expression as well as varied stem cell function (Reinke and Song, 2010). Interestingly, different TGFβ ligands have been shown to be differentially expressed during fetal wound healing. Notably, very low levels of TGFβ1 and TGFβ2, and high levels of TGFβ3 have been reported (Whitby and Ferguson, 1991; Ferguson and O’Kane, 1996). Furthermore, when exogenous TGFβ1 is applied to fetal wounds, scar formation reminiscent of adult wound healing is observed (Lin and Adzick, 1996). Building on this, it has been demonstrated that in adult cutaneous wounds reduction in TGFβ1 and TGFβ2 or exogenous application of TGFβ3 can reduce scar formation (Shah et al., 1995; Ferguson and O’Kane, 1996). TGFβ1 has been extensively reported to be pro-fibrotic and pro-inflammatory, (reviewed by Biernacka et al., 2011) and TGFβ2 has also been demonstrated to contribute to fibrosis promotion in numerous situations as well (Cordeiro et al., 1999; Hill et al., 2001). Conflicting evidence exists in regards to the role of TGFβ3 in scar formation as it has been reported that TGFβ3 is both pro, and anti fibrotic (Laverty et al., 2009). In regards to this, a recent clinical trial involving cutaneous
application of TGFβ3 for scar reduction failed to meet expected outcomes (Wu et al., 1997; Occleston et al., 2011). Activin ligands have also been shown to play a role in cutaneous wound healing (Werner and Alzheimer, 2006). Following wounding, both mice and humans demonstrate upregulation of activin A, however expression levels have not been examined in mammalian fetal wound healing.

These various observations in both mammalian scar-free fetal wounds and scar forming adult wounds allude to complex and poorly understood isoform specific effects of TGFβ/activin ligands in the wound healing process. Therefore, establishing the specific roles of different TGFβ/activin isoforms during scar-free wound healing and multi-tissue regeneration in adult amniotes is essential to our understanding of the differences in these programmes in adult humans and holds great promise in the field of regenerative medicine. Interestingly, some organisms retain the ability to heal wounds without scar formation into adult life. The next sections present an overview on our current understanding of post-natal scar-free, multi-tissue regeneration and the evidence of the role of TGFβ/activin signaling in the process.

Models of Multi-Tissue Regeneration

There exists a striking diversity of regenerative potential throughout Metazoa. Various invertebrates, such as planarians, are able to regenerate virtually every portion of the body (Sanchez-Alvarado, 2006). Among vertebrates, the regenerative potential is considerably more modest. However, various species are capable of replacing lost or damaged appendages and organs. Among the most commonly
recognized regeneration-competent species are: various teleost fish (e.g., zebrafish), which can regenerate portions of their heart, jaws, spinal cords and tail fins following various insults (Poss, 2007; Tal et al., 2010); *Xenopus* tadpoles, that can regenerate their tails, limbs and retina (Tseng and Levin, 2008); and urodele amphibians (newts and axolotls) that are able to regenerate their tails, limbs, spinal cords, and in some cases heart and retina (Brockes, 1984; Tsonis, 1996; Song and Stocum, 2010). Amongst amniotes, some of the most impressive examples of multi-tissue appendage regeneration come from lizards. Representative members of most major lineages, including polychrotids (e.g., *Anolis carolinensis*) and gekkotans (e.g., *Eublepharis macularius*) are able to regenerate their tail following loss of the appendage (Bellairs and Bryant, 1985; McLean and Vickaryous, 2011). Another notable example of amniote regeneration is digit tip regeneration, as demonstrated in rodents and primates (Illingworth, 1974; Vidal and Dickson, 1993; Rajnoch et al., 2003; Muneoka et al; 2008). Furthermore, several species of mice possess enhanced regenerative capacity including spiny mice (Acomys) (Seifert et al., 2012), MRL (Murphy Roths Large) mice (Herber-Katz, 1999; Leferovich and Herber-Katz, 2002), and mice with a point mutation in TGFβR1 (Liu et al., 2010).

Most examples of multi-tissue regeneration in vertebrates, with the possible exception of mouse and primate digit tips (Muneoka et al., 2008), begin with the formation of a mass of mesenchymal-like cells at the wound site (Wallace, 1981; Tsonis, 1996; Tamura et al., 2010). This cellular aggregation, commonly referred to as a blastema, is morphologically similar to an embryonic limb bud (Han et al.,
Blastema-mediated or epimorphic regeneration is characterized by an aggregation of proliferating cells that gives rise to replacement tissues (Carlson, 2007). Morphologically, the blastema appears to be composed of a homogenous population of undifferentiated cells. However, various recent studies have demonstrated that blastema cells are actually a heterogenous pool of lineage restricted progenitor cells (Brockes and Kumar; 2002; Kragl et al, 2009; Rinkevich et al., 2011; Lehoczky et al., 2011). Consequently, blastema cells are not a pluripotent (or perhaps even multipotent) population, but instead retain a memory of their germ layer origin (axolotls: Kragl et al., 2009; mouse digits: Rinkevich et al., 2011). Details of blastema formation remain poorly understood, but it is predicted that either reprogramming events occurring amongst the different lineage restricted progenitor cell populations that make up the blastema, or rapid expansion of tissue specific stem cell populations, or a combination of both processes give rise to the blastema (Kragl et al., 2009; Rinkevich et al., 2011; Jopling et al., 2011).

Throughout the process of epimorphic regeneration, the wound site and developing blastema is capped by a specialized wound epithelium. The wound epithelium first forms shortly after appendage loss, as original epidermal cells surrounding the wound quickly migrate across the site of injury (Stoick-Cooper et al., 2008). Once the wound area is covered, the wound epithelium begins to thicken, giving rise to an apical cap comparable with the apical ectoderm ridge (AER) observed during limb development (Christensen and Tassava, 2000; Nacu and Tanaka, 2011). This wound epithelium lacks the distinctive stratified appearance, basal keratinocyte polarity
and mature basal lamina observed in pre-wounding epidermis (Globus et al., 1980; Neufeld and Day, 1996). Matching its distinctive appearance, the wound epithelium demonstrates unique protein and gene expression profiles compared to normal epithelium (Han et al., 2005, reviewed by Campbell and Crews, 2008; Campbell et al., 2011; Monaghan et al., 2012). Numerous reports have established that the wound epithelium is vital for blastema induction and proliferation (Thornton, 1957; Christensen and Tassava, 2000; Nacu and Tanaka, 2011).

**TGFβ/activin and multi-tissue regeneration**

To date, aspects of TGFβ/activin signaling have been investigated in three different experimental models of multi-tissue regeneration: limb regeneration in axolotls (Levesque et al., 2007), tail regeneration in *Xenopus* (Ho and Whitman, 2008) and fin regeneration in zebrafish (Jazwinska et al., 2007).

*TGFβ signaling during axolotl limb regeneration*

As for other examples of epimorphic regeneration, axolotl limb regeneration begins with a period of wound healing followed by blastema outgrowth and tissue regeneration. Throughout axolotl limb regeneration *TGFβ1* mRNA expression is upregulated (Levesque et al., 2007). Injecting the small molecule inhibitor of TGFβ/activin signaling, SB-431542, into the limb stump delays the formation of wound epithelium, limits blastema cell proliferation and ultimately impedes
blastema formation (Levesque et al., 2007).

_TGFβ signaling during Xenopus tail regeneration_

Among _Xenopus_ tadpoles, all of the tissues of the tail including skin, neural tissue and muscle, can be fully regenerated following amputation. This regenerative capacity is restricted to tadpoles and diminishes and stops during metamorphosis (Slack et al., 2004). As has been demonstrated for models of epimorphic regeneration, genes active during embryonic development reactivate during _Xenopus_ tadpole tail regeneration (reviewed by Poss, 2010; Nacu and Tanaka, 2011). Within 15 minutes of tail amputation, there is an increase in phosphorylated SMAD2 (pSMAD2) immunostaining, suggesting activation of the TGFβ/activin pathway. Interestingly, this may be the earliest reported signaling event that occurs in regeneration (Ho and Whitman, 2008). This early pSMAD2 activation is restricted to a single layer of epidermal cells near the amputation site at the cut edge. However, as regeneration proceeds, pSMAD2 expression becomes more widespread, including many cells of the blastema, epidermis, and original notochord. Upregulation of TGFβ/activin family members _xTGFβ2_ (similar to TGFβ2), _xTGFβ5_ (similar to TGFβ1), _xGDF11_ and _xActivin-βA_ was observed using semi-quantitative PCR (Ho and Whitman, 2008). When SMAD2/3 phosphorylation was inhibited at the time of amputation (using the small molecule inhibitor SB-431542) both the wound epidermis and the blastema failed to form (Ho and Whitman, 2008). Furthermore, blockade of SMAD2/3 phosphorylation with SB-
431542 also appeared to inhibit other signaling pathways known to be activated during regeneration, including BMP and Erk (Ho and Whitman, 2008). The authors conclude that, TGFβ/activin signaling is required for the formation of the wound epithelium and the establishment of blastema that eventually constitute the regenerate appendage (Ho and Whitman, 2008).

*TGFβ signaling during zebrafish fin regeneration*

The most extensive investigation of TGFβ/activin signaling during multi-tissue regeneration comes from the study of zebrafish fin regeneration (Jazwinska et al., 2007). Using microarray analysis, the authors sought to determine which transcripts were upregulated at three different early time points following amputation: 0 hours, 6 hours (re-epithelialization completed) and 24 hours (start of blastema induction). The microarray screen revealed upregulation of *activin-βA* (Jazwinska et al., 2007). To confirm the microarray data, qRT-PCR was performed examining all the TGFβ family ligands known to signal through SMAD2/3 (viz. *TGFβ1-3, activin-βA and-βB, and GDF9-11*) at the same three early time points. Validating the microarray data, only the *activin-βA* transcript was significantly upregulated, increasing ~18 times at six hours post-amputation and 15 times at 24 hours, when compared to 0 hours time point. Using in-situ hybridization, *activin-βA* expression was localized to the interray pockets formed between the wound epidermis and blastema, and appeared to continue throughout regeneration (Jazwinska et al., 2007). Using the small molecule inhibitor SB-431542 the authors
further demonstrated that SMAD2/3 inhibition results in an abnormal wound epidermis and lack of blastema formation (as seen in *Xenopus* and axolotl; Levesque et al., 2007; Ho and Whitman, 2008). To further examine the signaling mechanism, the authors used a temperature sensitive mutant for fgf20a. Once heat shocked, these fish do not transcribe fgf20a and, as a result, a blastema fails to form following tail amputation (Whitehead et al., 2005). *Activin-βA* expression was still observed in the interray pockets of these mutants, suggesting that activin upregulation does not require FGF signaling, or blastema formation (Jazwinska et al., 2007). To elucidate the role of *activin-βA* in regeneration, knockdown morpholinos were used to ablate this gene (*activin-βA*), as well as the *Alk4* receptor gene. The result was a 50% and 80% reduction in regenerate tail size respectively suggesting other ligands may play redundant roles during regeneration.

*Limitations and unanswered questions*

Previous work has often focused on the use of the small molecule inhibitors SB-431542 and SB-505124. It is important to note however, that these molecules inhibit the phosphorylation of all canonical (SMAD) mediated TGFβ/activin signaling (i.e. both SMAD 2 and 3). Since SMAD 2 and 3 can be phosphorylated by either TGFβ or activin ligands, use of these inhibitors is not ligand specific. Observed differences in the upregulation of various TGFβ family ligands suggests that each may be playing unique and non-overlapping roles during regeneration and possibly in different species and different regenerating structures (Levesque et al., 2007;
Jazwinska et al., 2007; Ho and Whitman, 2008). Furthermore, activin-βA and Alk-4 knockdown morpholinos failed to completely recapitulate the effects of SB-431542 treatment, suggesting other TGFβ ligands besides just activin are playing roles during regeneration.

Together, these studies demonstrate that TGFβ plays a critical role during regeneration but do not conclusively indicate which ligands are responsible for driving the large induction of pSMAD2/3. Evidence from the study of zebrafish fin regeneration suggests that activin-A is the main ligand responsible for the pSMAD induction, but does not rule out other ligands. These findings suggest that the expression of various TGFβ family ligands should be further examined with highly sensitive techniques to clarify the differences seen in different models of multi-tissue regeneration. In particular, most work examining TGFβ/activin ligand expression has examined mRNA localization, thus work examining protein localization would complement the current data. Furthermore, the extremely rapid induction of TGFβ/activin–SMAD2/3 mediated signaling seen in Xenopus and zebrafish, combined with the loss of function studies, suggests that these unknown TGFβ/activin ligand combinations are playing important, instructive roles in dictating the initiation and success of regeneration. Once again, these evidences emphasize the need for a better understanding of the different roles played by individual TGFβ ligands in regeneration.
**Epithelial to mesenchymal transition**

One such role that various TGFβ/activin ligands could be mediating during multi-tissue regeneration is the epithelial to mesenchymal transition (EMT). The epithelial to mesenchymal transition is an evolutionary conserved process that occurs during development, wound healing, fibrosis and cancer metastasis. EMT involves the loss of epithelial phenotype and the acquisition of a mesenchymal phenotype (Nieto, 2011; Lim and Thiery, 2012). This transition is mediated by a set of transcription factors (Namely; Snail1, Snail2, Zeb1, Zeb2 and Twist1 and Twist2) that promote cellular motility. EMT transcription factors are upregulated by a variety of extracellular stimuli, including FGF, Wnt and TGFβ family signalling (Peinado et al., 2007; Lim and Thiery, 2012). Importantly, EMT is reversible and cells can cycle between epithelial and mesenchymal states via EMT, and the reverse process MET (mesenchymal to epithelial transition) (Lim and Thiery, 2012). Furthermore, EMT appears to be context dependent and occurs, much like SMAD signaling, within the framework of other signaling mechanisms such as fate induction and differentiation (Nieto, 2011; Lim and Thiery, 2012).

Throughout development, EMT occurs in successive waves during both morphogenesis and organogenesis playing roles in gastrulation, neural crest delamination, cardiac cushion and valve formation as well as palate fusion (Nashawd et al., 2004, Nieto, 2011; Lim and Thiery, 2012). In the adult, it has been proposed that EMT plays a role during wound healing and in support of this it has recently been shown that Snail2 is upregulated following injury to corneal epithelial
Despite this observation the role of the EMT in wound healing remains poorly understood and requires further examination. Currently, the best-studied examples of postnatal EMT involve pathological conditions such as fibrosis and cancer metastasis.

**EMT in fibrosis**

EMT has been suggested to be a potential driver of cardiac and renal fibrosis. Using transgenic lineage-tracing strategies it has been demonstrated that up to 30% of activated fibroblasts in fibrotic cardiac and renal tissue are of an apparent endothelial or epithelial lineage suggesting an EMT transition could underly their activation (Zeisberg et al., 2007; Zeisberg et al., 2008). EMT has also been shown to drive experimentally induced liver fibrosis and TGFβ induced lung fibrosis in several studies (reviewed by: Quaggin and Kapus, 2011). Despite these results, the role of EMT(s) during fibrosis remains a topic of considerable debate as a cause and effect relationship remains difficult to determine (see Quaggin and Kapus, 2011). Furthermore, EMT has also been suggested to play a role in the generation of cancer associated fibroblasts (CAF) which are described as activated fibroblasts that contribute to cancer progression and metastasis (Kalluri and Zeisberg, 2006).
**EMT in cancer**

TGFβ-induced EMT has been demonstrated to contribute to the metastasis of several cancer types including breast and head and neck cancer (Thiery et al., 2009; Heldin et al., 2012). EMT increases metastatic potential by converting stationary, transformed epithelial cells into motile, mesenchymal cancer cells (Thiery et al., 2009; Heldin et al., 2012). Over the past decade many studies have expanded the knowledge of EMT and metastasis in other tumor types and also corroborated the importance of TGFβ signaling in this process (reviewed by Heldin et al., 2012).

It has also become well established that EMT can confer other pro-metastatic properties to cancer cells in particular stem cell traits, including self-renewal capacity (Scheel et al., 2011). This was first demonstrated in immortalized human mammary epithelial cells (HMLE) where forced expression of EMT transcription factors Snail1 and Twist1 results in the acquisition of stem cell like characteristics (Mani et al., 2008). This work has rapidly been expanded and several important discoveries have been made including the observation that the cytokines TGFβ1 and Wnt5a when combined with a reduction in Wnt antagonists (Dickkopf-1 and secreted fizzled related protein 1) can induced a stem cell state through the EMT without any genetic manipulation of target cells (Scheel et al., 2011). Furthermore, EMT transcription factor Snail2 was demonstrated to cooperate with Sox9 to positively regulate the mammary stem cell state in nontransformed cells (Guo et al., 2012). This rapidly expanding pool of data that suggests EMT program can induce a
stem cell state has important implications for multi-tissue regeneration.

**EMT in regeneration**

EMT has never been definitively linked to multi-tissue regeneration, however there is a growing body of evidence that suggests it could be an important player in achieving regenerative capacity as well as determining the regeneration versus fibrosis balance. In models of multi-tissue regeneration EMT transcription factors have only been observed twice in the literature (Satoh et al., 2008; Kragl et al., 2012). Both of these observations involve the axolotl model of limb regeneration where it was demonstrated that different isoforms of the EMT transcription factor Twist are upregulated during regeneration (Satoh et al., 2008; Kragl et al., 2012). The authors of these studies do not mention EMT in their work, but do allude to the role of Twist during neural crest induction and delamination (Satoh et al., 2008; Kragl et al., 2012). The observation of EMT transcription factors during regeneration suggests a yet unknown role for these factors in the process. Speculating, with the current research in mind, it is likely that the roles EMT transcription factors play during regeneration include cell motility and cell fate determination. During regeneration, cells need to migrate to areas of tissue damage, thus the acquisition of cellular motility is likely a very important determinant of regenerative success. However, in models of blastema-mediated regeneration, a more striking need is the ability for progenitor pools to rapidly expand. It has been demonstrated in axolotls that this rapid expansion occurs through de-differentiation
of existing cells in a lineage restricted manner, and that pluripotent stem cells are not generated (Kragl et al., 2009; Rinkevich et al., 2011). Notably, this rapid expansion of progenitors is also blocked when TGFβ signaling is inhibited (Levesque et al., 2007; Jazwinska et al., 2007; Ho and Whitman, 2008). Aforementioned work in the cancer research field has demonstrated that the transfection of both normal and transformed mammary cells with EMT transcription factors Snail1, Snail2 or Twist causes a dedifferentiation and subsequent acquisition of stem cell like traits suggesting EMT transcription factors can act as lineage restricted reprogramming agents (Mani et al., 2008; Guo et al., 2012). Furthermore, recent work in drosophila has demonstrated that the Snail family transcription factor Worniu is continuously required in neuroblasts to maintain self-renewal by inhibiting premature differentiation (Lai et al., 2012). Mechanistically, it has been shown during muscle development that Snail binds particular E-boxes blocking differentiation promoting transcription factors from binding, thus in effect blocking differentiation and promoting progenitor phenotypes (Soleimani et al., 2012). Taken together, it is tempting to speculate that EMT transcription factors, if induced by the correct extracellular stimuli, could cause a subset of cells to dedifferentiate and become a lineage restricted, tissue specific progenitors that could drive regeneration.

**The leopard gecko (Eublepharis macularius) model of scar free multi-tissue appendage regeneration;**

Mentioned previously, many lizards such as *Eublepharis macularius* and *Anolis*
*carolinensis* can undergo post amputation regeneration of their tails following the voluntary shedding of their tails in a process term autotomy (Bellairs and Bryant, 1985). Tail autotomy has developed evolutionarly as an anti-predation strategy and has been extensively examined in terms of ecological costs and benefits (Naya et al., 2007). More recently, tail autotomy and subsequent regeneration has begun to be examined as a model of scar-free-multi-tissue appendage regeneration. To date, most research examining multi-tissue regeneration in vertebrates has focused on anamniotes such as *Xenopus*, zebrafish and axolotls. Models studying amniote regeneration are however becoming more common with digit tip regeneration and ear whole punch closure being studied in mice, primates (such as humans) and rabbits (Bielefeld et al., 2012). One of the major benefits of the *Eublepharis macularius* model of multitissue regeneration is that the complete regeneration of a large, complex appendage such as the tail, has not been reported in any other amniotes (McLean and Vickaryous, 2011). Furthermore it has been observed that tail regeneration in the leopard gecko involves the formation of a mass of non-differentiated, highly proliferative cells resembling the blastema seen frequently in anamniotes models of regeneration (McLean and Vickaryous, 2011).

The regenerative response following tail loss in the leopard gecko has been documented to follow a conserved series of morphological and histological events that have been characterized and developed into a staging system (McLean and Vickaryous, 2011). The original gecko tail is a complex, multi-tissue appendage that contains numerous tissue types including striated muscle, adipose tissue,
vasculature, a bony vertebral column, spinal cord, notochord, epidermis, and dermis and can represent up to 41% of total body length (McLean and Vickaryous, 2011). Furthermore, the gecko tail has unique adaptations designed to facilitate autotomy including intervertebral fracture planes and proximal vascular sphincters to reduce blood loss (McLean and Vickaryous, 2011).

Post autotomy morphology: Wound healing: stages I-III

Immediately following tail loss (Stage I) the amputation plane is an open wound with various tissues exposed including vertebrae, spinal cord, dermis, muscle, and adipose tissue. Due to the arterial sphincters there is relatively little blood loss. From 18 hours to up to 8 days post autotomy (Stage II) an exudate clot containing tissue debris, erythrocytes and serum forms over the exposed tissues. Notably, during this stage epidermal cells being to migrate across the wound, deep to the exudate clot, to form what will become the wound epidermis. These epidermal cells are proliferative (PCNA positive). Deep to the wound epidermis and distal to the spinal cord, another population of cells is also demonstrated to be proliferative, possibly indicating early blastema cells. Stages I to III are grouped together as the wound healing phase (McLean and Vickaryous, 2011). From 4 days to 8 post autotomy the loss of the exudate clot is observed marking stage III of regeneration. During this stage no regenerative outgrowth is observed, however, the wound epithelium covers the entire wound surface and begins to thicken and stratify suggesting the formation of the apical ectodermal cap (AEC). Interestingly,
epidermal protrusions (epidermal downgrowths) also begin to appear lateral to the wound edges, which are highly proliferative, migrate proximally and frame the blastema, which by the end of stage III is also highly proliferative and well-established, deep to the wound epithelium and distal to the spinal cord.

Post autotomy morphology: Regeneration: Stages IV-VII

Following stage III, from 8 to 15 days post autotomy there is a pronounced outgrowth of the regenerative tail (stage IV). During this stage, the blastema becomes a large and pronounced mass of proliferative cells that constitutes most of the outgrowth seen. Notably, the wound epidermis becomes much thicker than normal, adjacent epidermis and the epidermal downgrowths become more prominent. Furthermore, stage IV marks the initiation of regenerative myogenesis with condensations of myoblasts appearing to be more differentiated in a proximal (most differentiated) to distal (least differentiated) pattern. From day 12 to 26 post autotomy (stage V) the tail continues to lengthen and regenerate. Early in stage V, the blastema remains prominent but differentiation appears to be occurring in a proximal to distal fashion as a cone-like condensation of putative cartilage cells begin to deposit ECM components and components of the nervous tissue such as axons appear. Myogenesis continues to occur in a proximal to distal pattern, with regenerate myotubes becoming more distinct. Towards the end of stage V, the wound epithelium begins to become keratinized and undergoes scalation. Differentiation in the blastema becomes more pronounced with presumptive
cartilage cells differentiating into Sox9 expressing chondrocytes and chondroblasts again in a proximal to distal gradation (McLean and Vickaryous, 2011). From 18 to 30 days post autotomy (stage VI) the regenerate tail continues to differentiate with the wound epidermis becoming the same thickness as normal epithelium and most blastema cells differentiating into numerous, defined tissues. Finally, from day 25 onward (stage VII) the regeneration program becomes complete, with the regenerate tail achieving a tapered shape and pigmentation, and most blastema cells differentiated. The fully regenerate tail, though similar to the original structure, is not a perfect replica as the skeletal elements of the original tail are replaced by a cartilaginous cone and, importantly, do not undergo skeletal mineralization (McLean and Vickaryous, 2011). Despite the extensive morphological and histological characterization, tail regeneration in Eublepharis macularius has not been extensively examined at the molecular level. Preliminary analysis of the scar free wound healing process has demonstrated that TGFβ3 is present only late (stage V onward) during gecko tail regeneration and is localized to chondrocytes of the regenerating cartilaginous cone (McLean and Vickaryous, 2011; Delorme et al., 2012).
**Rationale**

TGFβ/activin signaling has been demonstrated to play a variety of important roles during both scar formation and scar-free wound healing leading to regeneration. Available evidence strongly indicates that the reparative outcome (scarification versus regeneration) is determined by the spatiotemporal expression of various TGFβ and activin ligands. The goal of this research was to determine the stage of onset and location of expression of various TGFβ/activin signaling ligands and potential downstream targets during the process of tail replacement using a regeneration-competent amniote model, the leopard gecko (*Eublepharis macularius*).

I hypothesize that ligands of the TGFβ/activin signaling pathway play distinctive roles in different cell populations and at different stages of the scar-free wound healing and tail regeneration program.

**Objective 1**

To establish that the canonical (SMAD-mediated) TGFβ/activin pathway is activated during gecko tail regeneration and determine if the blastemal cell population is actively responding to TGFβ/activin signaling by documenting the onset of induction and cellular localization of phosphorylated SMAD2 (pSMAD2), and its potential co-localization with the proliferation, and subsequent blastema marker PCNA, using western blot analyses and immunofluorescence.
Objective 2
Investigate the expression and localization of TGF\(\beta\)/activin family members capable of activating the TGF\(\beta\)/activin signaling pathway during gecko tail regeneration. More specifically, by determining protein and mRNA expression of TGF\(\beta\)1, TGF\(\beta\)2, TGF\(\beta\)3 and activin-\(\beta\)A through the use of western blotting, immunohistochemistry and qRT-PCR. This includes the determination of gecko mRNA sequences (unavailable to date) for each of TGF\(\beta\)1, TGF\(\beta\)2, TGF\(\beta\)3 and activin-\(\beta\)A through PCR based approaches.

Objective 3
To investigate the potential role of novel TGF\(\beta\)/activin induced cellular programs in particular, the epithelial to mesenchymal transition (EMT) during gecko tail regeneration (stages III-VI) by determining the expression pattern of EMT transcription factors known to be induced by TGF\(\beta\)/activin signaling, specifically Snail1, Snail2, and Zeb 2 using qRT-PCR.
Material and Methods

A list of recipes for solutions prepared and suppliers for chemicals are found in appendices I, and II, respectively. Primers used for PCR reactions can be found in Tables 1 and 2 in appendix III.

Animal Care and Handling

All animals were captive bred and obtained from a commercial supplier (Global Exotic Pets, Kitchener, Ontario). Leopard geckos were housed individually in standard rat enclosures in the Central Animal Facility at the University of Guelph, following husbandry protocols of McLean and Vickaryous (2011) and standard animal care procedures in accordance with the Canadian Council on Animal Care (CCAC) Guidelines (Animal utilization protocol numbers 09R026 and 10R113, approved by the University of Guelph Animal Care Committee).

Tail Collection and Tissue Segmentation

Tail collection was achieved by autotomy using the protocol of McLean and Vickaryous (2011). Briefly, leopard geckos were manually restrained and tails were firmly pinched at a position halfway between the cloaca and tail tip. Following pinching, the distal portion of the tail is self-detached, whereas the proximal portion is retained. Following autotomy, tails were allowed to regenerate to appropriate stages and autotimized a second time. Secondarily detached tails include the regenerated tissues plus a portion of original tail. Following the second autotomy,
Tail were segmented (by transverse cuts using a #15 blade scalpel blade) into three domains: regenerated tissue; a segment (~1.0 cm long) of original tissue immediately adjacent to the regenerated tissue (‘distal tissue’); and the next consecutive segment of original tissue more proximal to the body (‘proximal tissue’). Each segment was immediately flash frozen in liquid nitrogen and stored at -80 for protein and RNA extraction.

**Western Blot analysis**

Protein was extracted from each segment of individual autotomized tails and homogenized in protein lysis buffer (Cell Signaling), supplemented with 1mM PMSF, 2µg/ml aprotinin (Sigma-Aldrich), 1mM Na$_3$VO$_4$ (New England Biolabs) and 1% phosphatase inhibitor cocktail 2 (Sigma-Aldrich). For each segment, a 30 µg sample of total protein lysate was resolved on 10% and 15% polyacrylamide gels under reducing (pSMAD2, SMAD2, α-Tubulin) or non-reducing conditions (TGFβ1) and subsequently transferred to PVDF membranes (Roche). Membranes were first blocked for 1 hour in TBST containing 5% skim milk powder and then incubated with primary antibody diluted in blocking solution overnight at 4°C. The concentrations of primary antibodies were as follow: 1:1,000 rabbit monoclonal anti-pSMAD2 (Ser465/Ser467) (Cell Signaling), 1:1,000 mouse monoclonal anti-SMAD2 (Cell Signaling), 1:500,000 mouse monoclonal anti-tubulin (Sigma-Aldrich), 1:1,000 rabbit polyclonal anti-TGFβ1 (Santa Cruz). Membranes were then washed with TBST, incubated with the appropriate HRP-conjugated secondary antibodies for 1 hour at room temperature, washed again with TBST and developed with HRP.
chemiluminescent substrates (Millipore) visualized on a ChemiDoc XRS (Bio-Rad). Three biological replicates were examined by western blotting for each protein of interest.

**Immunofluorescence and Immunohistochemistry**

Fixation and sectioning were performed as previously described (McLean and Vickaryous, 2011). Briefly, tissues were fixed in either 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) and serially sectioned. Slide mounted serial sections were then deparaffinized and rehydrated. Sections destined for immunohistochemical analysis were quenched in 3% H2O2 for 30 minutes. All sections were rinsed with phosphate buffered saline (PBS). Next, heat induced epitope retrieval (citrate buffer heated to 90°C for 12 minutes) was used to unmask antigenic sites. Following antigen retrieval, sections were incubated with appropriate blocking reagent for one hour, followed by primary antibody overnight at 4°C. Primary antibody concentrations were 1:150 anti-pSmad2 (Ser 465/467) (Cell Signalling), 1:300 anti-PCNA (Dako), and 1:500 anti-TGFβ1 (Santa Cruz).

Primary antibody was omitted from one section on each slide to function as a negative control. For immunohistochemistry, slides were rinsed in PBS and then all sections were incubated with appropriate biotinylated secondary antibody (Vector Laboratories) at a concentration of 1:500. Slides were then incubated with peroxidase-streptavidin substrate (Vector Laboratories) for 1hr at room temperature, and visualized with 3-3’- diaminobenzidine (DAB from Vector Laboratories; diluted in 5ml dH2O, for 6 minutes per slide). Slides were then rinsed in dH2O, counterstained in Mayers Hematoxylin for 4 minutes and mounted with
coverslips. For immunofluorescence, secondary antibody concentrations and incubations were 1:300 Alexa-Fluor goat anti-rabbit 488 (Life Technologies), or 1:300 Cy3 Goat anti-mouse (Jackson ImmunoResearch Laboratories) for one hour at room temperature. Following the addition of fluorescent secondary antibodies, slides were incubated with 0.3 μM 4’,6’-diamidino-2-phenylindole (DAPI)(Sigma) diluted in PBS for 4 minutes, washed twice and mounted with aqueous mounting media and coverslips.

Identification of Eublepharis macularius cDNA sequences using degenerative PCR
RNA was isolated from segmentated tails frozen at -80 by homogenization in Trizol (Life Technologies) at cold temperature followed by the Aurum total isolation RNA kit (Bio-Rad). cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad) with 1mg total RNA. Degenerate primers were designed by eye based on conserved sequences identified using ClustalW (EMBL-EBI) and publicly available sequences from related species. (For primer sequences see Tables 1 and 2). PCR was performed on a CFX-96 thermocycler (Bio-Rad) using SYBR Green I Supermix (Applied Biosystems) across various temperatures. Amplicons were run on a 2% agarose gel and correctly sized bands were subsequently extracted using illustra GFX PCR Gel Band Purification Kit (GE Healthcare). Following extraction, amplicons were either sent for sequencing at the advanced analysis facility of the University of Guelph or cloned into a TOPO-TA cloning vector pGEM-T Easy (Promega) and then sequenced. Sequence information was then analyzed using NCBI nucleotide blast and ClustalW (EMBL-EBI) to confirm sequence specificity (Table 3). Identified
sequences were deposited to NCBI GenBank (numbers to be added once sequences annotated).

**qRT-PCR analysis**

RNA was isolated and cDNA was produced as described above. Appropriate quality control steps were included along the way conforming to the MIQE guidelines (Bustin et al., 2009; Taylor et al., 2010). The cDNA that was obtained was diluted 1:4 in nuclease free water and 4 µl of diluted cDNA was added to reaction mixes (10 µl) containing 5 µl Sso Fast Eva Green Supermix (Bio-Rad) and 500 nM of each primer. Primers were designed using Primer3 (NCBI) and optimized using a temperature gradient and eight point standard curve to determine PCR efficiency. Acceptable efficiency was deemed between 90% and 110%. All amplicons were determined to be specific by agarose gel analysis and subsequent sequencing. qRT-PCR amplifications were carried out using a CFX-96 (Bio-Rad) as follows: an initial denaturation step 2 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C and 5 seconds at 59°C. Data was expressed as relative gene expression normalized to 18s mRNA which was determined to be a suitable housekeeping gene using qBase Plus software (BioGazelle). Kruskal-Wallis non-parametric statistics were performed on qRT-PCR data followed by a Dunns post test with a p-value cutoff of >0.05. Three biological replicates and three technical replicates were used for all qRT-PCR experiments.
Results

*TGFβ/activin signaling is active in the regenerate blastema.*

The blastema is characterized by a highly proliferative population of cells contributing to the formation of replacement tissue (Stoick-Cooper et al., 2007; Stocum and Cameron, 2011). To determine if cells compromising the presumptive blastema are actively responding to the TGFβ/activin signals we performed dual immunofluorescence with pSMAD2 and the proliferation marker proliferating cell nuclear antigen (PCNA) at several stages of tail regeneration.

During the late wound healing phase of tail regeneration (stage III), presumptive blastema cells form as an aggregation of mesenchymal like cells at the distal end of the torn spinal cord (Fig. 1. A). In stage III regenerate tails PCNA labels this same population of mesenchymal like cells distal to the torn edge of the spinal cord representing the initiation of the blastema (Fig. 1. B). Isolated PCNA positive cells are also detected in the regenerate dermis lateral and distal to the spinal cord (Fig. 2.). pSMAD2 expression appears in numerous nuclei of cells within the spinal cord as well as a large portion of cells in the initial blastemal aggregation (Fig. 1. C). PCNA and pSMAD2 co-localize to numerous cells within the PCNA positive blastema located distal to the spinal cord suggesting active TGFβ/activin signaling within early blastema cell populations (Fig. 1. D)

Following the wound healing phase (stage III) the regenerating tail begins to grow
distally and form a cone-like outgrowth often referred to as the regenerate tail bud (stage IV). This outgrowth is mostly attributed to a large increase in the number of mesenchymal like blastema cells located distal to the spinal cord (stage IV) (Fig. 1. E). PCNA labels a large portion of these blastema cells supporting the definition of the gecko regenerate blastema as a proliferate pool of mesenchymal like cells (Fig. 1. F). Interestingly, the regenerate epithelium appears to lack a basally restricted proliferative population and has become strikingly hyperthickened as it resembles a structure known as the wound epithelium (Fig. 1. F). pSMAD2 nuclear localization appears in most cells located in the regenerate tail bud suggesting extensive TGFβ/activin signals within early regenerate tail tissues (Fig. 1. G). Most PCNA positive cells within the regenerate tail bud double label for pSMAD2 suggesting that blastema cells are actively responding to TGFβ/activin signals in early regenerate tissues (Fig. 1. H)

As regenerate tail outgrowth continues (stage V) the blastemal cell population further increases in size to support tail growth (Fig. 1. I). PCNA labels most cells throughout the regenerate tail bud demonstrating a large blastema proximal to the wound epithelium (Fig. 1. J). pSMAD2 staining again appears robust throughout the tail bud further suggesting strong TGFβ/activin signals (Fig. 1. K). Closer inspection of the regenerate tail bud reveals numerous dual positive cells indicating again that blastema cells are actively responding to TGFβ/activin signals (Fig. 1. L)

As the regenerative outgrowth begins to slow down (stage VI) tissue differentiation
begins to become more apparent. Particular tissues, such as regenerating muscle and cartilage, become highly visible in distal regenerate tissues at stage VI (Fig. 1. M). PCNA expression becomes much more localized to tissue specific compartments in stage IV tails such as lateral cartilaginous cells of the cartilage cone (Fig. 1. M). Fewer PCNA positive cells are also observed in the dermal compartment further supporting blastemal cell differentiation (Fig. 1. N). Furthermore, the distal wound epithelium is no longer hyperthickened, suggesting maturation of wound epithelium to normal epithelium. pSMAD2 staining remains robust throughout the regenerating tail bud likely owing to the important role of TGFβ/activin signaling in cartilage development and tissue differentiation (Fig. 1. O). PCNA and pSMAD2 appear to co-localize to fewer cells within the mesenchymal compartment of the regenerate tail, supporting the notion of blastemal differentiation at stage VI (Fig. 1. P). These observations demonstrate that TGFβ/activin signals are highly active throughout the regenerate tail bud in early stages of tissue regeneration and that blastema cells are actively responding to these TGFβ/activin based signals.

*Phosphorylated-SMAD2 and PCNA expression in original and regenerate epidermis*

During most instances of multi-tissue regeneration a unique structure known as the wound epithelium forms distal to the blastema. In the leopard gecko this wound epithelium becomes apparent at stage II and stage III where epithelial cells migrate to cover the autotomy wound and subsequently undergo proliferation. Stage III wound epithelium is normal thickness but demonstrates a lack of a basally
restricted PCNA population of keratinocytes (Fig. 2. A) pSMAD2 expression is detected throughout the wound epithelium at stage III (Fig. 2. B). Moving to stage IV the wound epithelium becomes hyperthickened with PCNA labeling a population of suprabasal keratinocytes (Fig. 2. C). pSMAD2 expression is detected throughout the epithelium but appears to be absent in the most basal layers (Fig. 2. D). Stages V reveals a hyperticked epithelium similar to stage IV with PCNA and pSMAD2 being expressed by suprabasal keratinocytes (Fig. 2. E, F). As regeneration proceeds (stage VI) tissue differentiation beings to occur and the wound epithelium becomes normal thickness. PCNA begins to be expressed in basal keratinocytes of the stage VI epithelium and pSMAD2 is observed suprabasally (Fig. 2. G, H). Original epithelium in the leopard gecko demonstrates a basally restricted PCNA population (Fig 2. I) as well as pSMAD2 expression detected throughout the epidermis (Fig. 2. J). Interestingly, examining the PCNA expression in the lateral aspects of a stage V tails reveals the point at which the normal epidermis and wound epidermis transition (Fig. 2. L). Moving left to right (Fig. 2. L), PCNA expression is initially expressed by basal keratinocytes but rapidly becomes suprabasal moving towards the regenerate tissue. These results reveal that expression of PCNA and pSMAD2 in the wound epithelium of the leopard gecko is dynamic and different than that of the original epithelium.

*Phosphorylated-SMAD2 is upregulated in regenerating tails.*

To confirm that the canonical TGFβ/activin pathway is activated during tail regeneration, we investigated the protein expression of pSMAD2, native SMAD2 as
well as the TGFβ ligand TGFβ1 using western blotting of segmented regenerate tails (α-tubulin used as a loading control) (Fig. 3. A-D). In stage III, IV, V and VI tails pSMAD2 is detected most strongly in the distal regenerate tissue confirming robust activation of the canonical TGFβ/activin signaling pathway in the tissues of the regenerate tail bud and blastema (Fig. 3. A-D). pSMAD2 is not detected in original tail tissue and is only weakly observed in more proximal tissues sections (Fig. 3. A-D). Native SMAD2 is expressed relatively evenly across all tissues examined, as is our loading control α-tubulin (Fig. 3. A-D). TGFβ1 appears to be expressed relatively evenly across all tissues with the notable exception of the most distal regenerate tissue in stage IV tails (Fig. 3. B). These observations confirm that pSMAD2 is strongly induced in all distal regenerate tissues further demonstrating robust TGFβ/activin signaling in the regenerate tail bud. This data also suggests that TGFβ1 may not be the pSMAD2 inductive ligand, in particular at early stages of regeneration (stage IV).

*TGFβ1 expression during tail regeneration.*

To further confirm this conspicuous absence of TGFβ1 at stage IV we performed immunohistochemistry for TGFβ1 at numerous stages of regeneration (stage III, IV, V, VI) and original tail tissue (Fig. 4). In original tissue from both proximal tail sections as well as non-regenerate tails TGFβ1 expression is observed strongly throughout the epidermis (Fig. 4. A). TGFβ1 expression is also observed surrounding numerous mesenchymal-like dermal cells (Fig. 4. A: cells identified
with M and arrows). In stage III regenerate tissue TGFβ1 expression is observed weakly in the regenerate wound epidermis as well as in a variety of putative immune cells located in the dermis proximal to the autotomy plane (Fig. 4. B: cells identified with I and arrows). Strikingly, in stage IV regenerate tissue TGFβ1 expression is nearly absent from the wound epidermis as well as the proximal blastemal population confirming our western blot data in Figure 3 (Fig. 4. C). As regeneration proceeds (stage V) TGFβ1 expression begins to be observed in lateral aspects of the wound epithelium as well as surrounding numerous cells within the blastema (Fig. 4. D). By stage VI TGFβ1 expression has returned to the maturing wound epithelium (Fig. 4. E). Furthermore, the expression of TGFβ1 by cells within the stage VI dermis beings to resemble that of original tissue (Fig. 4. E). The immunohistochemical localization of TGFβ1 during tail regeneration further confirms the observation that TGFβ1 is largely absent from early (stage IV) regenerate tissue, and as regeneration proceeds TGFβ1 expression gradually returns to as regenerate tissues differentiate.

*Non-overlapping TGFβ1 and pSMAD2 expression in early tail regeneration.*

The observation that TGFβ1 is absent in early regenerate tissue despite the large amount of pSMAD2 being present suggests the presence of other TGFβ/activin ligands. To confirm the non-overlapping expression of TGFβ1 and pSMAD2 we examined the expression of TGFβ1 and pSMAD2 on adjacent tissue sections (Fig. 5). In stage IV regenerate tails a structure known as the epidermal downgrowth forms
and appears to separate original tissue from the regenerate blastema (McLean and Vickaryous, 2011) (Fig. 5). On the lateral side of the epidermal downgrowth TGFβ1 expression is observed in the epithelium as well as numerous dermal cells (Fig. 5. A, B). However, on the medial side of the epidermal downgrowth TGFβ1 expression is undetectable in regenerate tissues (Fig. 5. A, C). pSMAD2 expression is detected in numerous TGFβ1 expressing cells of the epidermis and dermis within the original tissue lateral to the epidermal downgrowth (Fig. 5. D, E). Interestingly, in regenerate tissues medial to the epidermal downgrowth where TGFβ1 expression is not observed there appears to be a large increase in the number of pSMAD2 positive cells (Fig. 5. D, F). This result confirms the reciprocal expression of TGFβ1 and pSMAD2 in original and regenerate tissues and suggests another TGFβ/activin ligand is present in early regenerate tissues.

Activin-βA is upregulated in the blastema during early stages of tail regeneration.

To identify the unknown pSMAD2 inducing ligand(s) at stage IV of regeneration we performed a qRT-PCR screen for pSMAD2 activating ligands in regenerate tissue from stage IV blastemas. To perform this screen we first cloned all three members of the TGFβ/activin family, TGFβ1, TGFβ2, TGFβ3 as well as activin-βA which are known to classically activate pSMAD2 as well as the TGFβ superfamily member bone morphogenetic protein 2 (BMP2) (Moustakas and Heldin, 2009). We then examined mRNA expression of these pSMAD2 activating ligands using qRT-PCR on segmented stage IV tails. Comparing proximal, distal and regenerating tissue at stage IV, we
found that mRNA expression of TGFβ1 and remains relatively constant (Fig. 6) demonstrating no transcriptional upregulation is taking place in the newly forming tissues and further confirming our western blot and immunohistochemistry data on TGFβ1. Although TGFβ2, TGFβ3 and BMP2 reveal slight upregulation in regenerating tissues with TGFβ3 being the largest, these fold increases are not statistically significant (Fig. 6). We did however observe a significant 15-fold upregulation in activin-βA mRNA expression in regenerating tissues (Fig. 6). These results point towards activin-A as a candidate ligand responsible for the large induction of pSMAD2 during the early stages of tail regeneration.

Identification of novel TGFβ/activin target genes upregulated in regenerate tissues.

TGFβ/activin ligands participate in a wide range of cellular processes, many of which are likely to play important roles during multi-tissue regeneration. We investigated the TGFβ/activin induced target genes Snail1, Snail2 (formerly Slug) and Zeb2 at stage IV tails using qRT-PCR. Snail1, Snail2 and Zeb2 are transcription factors known to mediate the epithelial to mesenchymal transition (EMT) a process implicated in driving cell motility as well as stemness (Lim and Thiery, 2012). The EMT process has been implicated in development, cancer and wound healing but has never been suggested to play a role in multi-tissue regeneration. Interestingly we observed a significant upregulation of Snail1 and Snail2 (but not Zeb 2) in the regenerate tissues of the gecko tail compared to regenerate tissues. (Fig. 7). This novel finding combined with our robust pSMAD2 expression seen in the blastema
suggests that the epithelial to mesencymal transition could be an important, TGFβ/activin mediated process that is active during multi-tissue regeneration.
Figure 1. Immunofluorescence of PCNA (red) and pSMAD2 (green) expression during four stages of gecko tail regeneration and associated schematic representations of longitudinal sections. Stages III (A-D) dotted line surrounds putative blastema cell population distal to spinal cord. Stage IV (E-H). Stage V (I-L). Stage VI (M-P) dotted line surrounds cartilaginous cone. All images taken at 20x magnification, scale bars = 100 µm. sc= spinal cord. cc = cartilaginous cone.
Figure 2. Immunofluorescence of PCNA (red) and pSMAD2 (green) expression in distal wound epithelium and blastema during four stages of tail regeneration, original tissue, and stage V transitional epithelium. Stage III (A-B). Stage IV (C-D). Stage V (E-F). Stage VI (G-H). Original tissue (I-J). Stage V transitional epithelium (L-M), arrow marks transition between original epithelium (left side) and wound epithelium (right side). All images taken at 40x magnification, scale bars = 200µm. bv = blood vessel, cc = cartilaginous cone.
Figure 3. Representative western blots analysis of segmented gecko tails at four stages of tail regeneration as well as tissue from original tails (n = 3). Stage III (A). Stage IV (B). Stage V (C). Stage VI (D). Note the induction of pSMAD2 in most distal regenerate tissue at all stages of regeneration and the conspicuous absence of TGFβ1 protein expression in stage IV regenerate tail tissue. Native SMAD2 and α-tubulin remain relatively constant and serve as loading controls.
Figure 4. Immunohistochemical localization of TGFβ1 protein expression during four stages of gecko tail regeneration as well as original tissue, positive signal is demonstrated by brown staining (DAB chromogen). Negative control tissue shown on the right hand panel. Original tissue (A, An). Stage III (B, Bn). Stage IV (C, Cn). Stage V (D, Dn). Stage VI (E, En). Scale bar = 100 µm. Arrows marked M = TGFβ1 positive mesenchymal like cells. Arrows marked I = TGFβ1 positive immune cells. OE = Original epithelium. WE = Wound epithelium.
Figure 5. Non-overlapping expression of TGFβ1 and pSMAD2 in original and regenerate tissue in stage IV regenerate tails. TGFβ1 expression (A) as well as high magnification insets (B, C) and negative controls (B^n, C^n). pSMAD2 expression on adjacent tissue section (D) as well as high magnification insets (E, F). Scale bar for (A, D) = 200 µm, scale bar for (B, C, E, F) = 50 µm.
Figure 6. Relative mRNA levels of TGFβ family members in segmented stage IV regenerate tails determined using quantitative real-time polymerase chain reaction (qRT-PCR) and normalized to 18s mRNA levels. Graph depicts the average fold change (±SEM) in segmented tissue sections relative to most the proximal tissue section. A statistically significant increase in activin-βA was observed in the most distal tissue section corresponding to newly regenerate tissue and the position of the blastema. Statistical significance was determined using Kruskal-Wallis non-parametric statistics followed by a Dunns post test with a p-value cutoff of p >0.05. n = 3 for biological and technical replicates.
Figure 7. Relative mRNA levels of EMT transcription factors in segmented stage IV regenerate tails determined using quantitative real-time polymerase chain reaction (qRT-PCR) and normalized to 18s mRNA levels. Graph depicts the average fold change (±SEM) in segmented tissue sections relative to most the proximal tissue section. A statistically significant increase in Snail1 and Snail2 was observed in the most distal tissue section corresponding to newly regenerate tissue and the position of the blastema. Statistical significance was determined using Kruskal-Wallis non-parametric statistics followed by a Dunns post test with a p-value cutoff of p >0.05. n = 3 for biological and technical replicates.
Discussion

The TGFβ/activin signaling pathway is activated throughout the tail regeneration program.

Among amniotes, one of the most dramatic examples of spontaneous multi-tissue regeneration is the restoration of the lizard tail. Following tail loss (typically in response to the threat of predation), many lizard species are able to recreate a replacement appendage, complete with skeletal support, nerves, muscle and a spinal cord (Bellairs and Bryant, 1985). Previous work in anamniotes, namely, zebrafish, axolotl and Xenopus has investigated the TGFβ/activin signaling events that occur during early stages of tail fin regeneration (Jazawinska et al., 2007; Levesque et al., 2007; Ho and Whitman, 2008). We determined that the TGFβ/activin signaling pathway is also being activated during tail regeneration in geckos. TGFβ/activin signaling occurs throughout blastema formation, regenerative outgrowth and tissue differentiation, representing stages III-VI of the regeneration program. Phosphorylation and subsequent nuclear import of SMAD2 occurs following either TGFβ or activin ligands binding to their cognate receptors (Moustakas and Heldin, 2009). The nuclear localization of pSMAD2 with immunofluorescence serves as robust readout of active TGFβ/activin signalling within a cell. Our immunofluorescence data (further confirmed by western blot) demonstrates that pSMAD2 expression is robust throughout the regenerating tissues of the regenerate tail bud. As evidenced by immunofluorescent co-localization of PCNA
and pSMAD2, we demonstrate that a large portion of putative blastema cells are actively responding to TGFβ/activin signaling throughout regeneration.

Interestingly, TGFβ/activin ligands are classically thought of as antiproliferative/cytostatic cytokines as SMAD2/3 complexes are known to inhibit cell cycle progression (Sandhu et al., 1997; Seoane et al., 2004; Alexandrow and Moses, 1995). However, during development as well as cancer progression the cytostatic functions of TGFβ signaling can be overcome by a number of different mechanisms (Massague, 2012). Cellular context, in particular, crosstalk with other signaling pathways has been demonstrated to be able to overcome the TGFβ mediated cytostatic program through integration at the level of the SMADs (Guo and Wang, 2009; Massague, 2012). For example, SMAD-β-catenin complexes driven by TGFβ and Wnt signalling promote stem cell proliferation and self-renewal by stimulating mitosis, while inhibiting differentiation (Jian et al., 2006). Thus, the ability for SMAD proteins to cooperate with other signals and direct diverse transcriptional effects depending on the cellular context represents a logical explanation for their consistently observed involvement in multi-tissue regeneration.

Activin, and not TGFβ is upregulated.

pSMAD2 expression is a reliable readout of canonical TGFβ/activin signaling (Moustakas and Heldin, 2009), but does not distinguish between activating ligand(s). Given the documented roles of TGFβ1 during mammalian wound healing
(Ferguson and O’Kane, 1994; Bielefeld et al., 2012), we investigated the spatio-temporal expression of TGFβ1 during gecko tail regeneration. Our western blot data demonstrates that TGFβ1 is constitutively present in original tail tissue and at most stages of regeneration (stages III, V-VI). However, detectable TGFβ1 was conspicuously absent from regenerating tissues at stage IV. We further confirmed this result using immunohistochemistry, where we observed that TGFβ1 protein expression is strikingly absent from the early regenerate tissues, including the blastema, and wound epidermis. This unusual result suggests that the absence of TGFβ1 from early regenerate tissues may be a requirement for successful regeneration. TGFβ1 is classically characterized as a pro-fibrotic cytokine. In agreement with this, scar-free wound healing in fetal mammals is characterized by lower levels of TGFβ1 and higher levels of TGFβ3, when compared to scar forming wounds, while in adult mammals scar formation is driven, at least in part, by an increase in TGFβ1 (Soo et al., 2003; Larson et al., 2010; Bielefeld et al., 2012). Furthermore, when scar-free fetal wounds are treated with TGFβ1, scarification occurs (Lin and Adzick, 1996). Thus, the limited expression of this protein is consistent with tail regeneration as a scar-free phenomenon and suggests that the absence of TGFβ1 in early stages of regeneration may be a requirement for scar-free wound healing.

Despite the absence of TGFβ1 expression in regenerating tissue we still observed a large proportion of cells of the blastema and wound epithelium actively responding to TGFβ/activin signals as evidenced by nuclear pSMAD2. In order to investigate the
ligands responsible for this SMAD2 phosphorylation in regenerate tissue we used qRT-PCR to screen for TGFβ/activin mRNA transcripts upregulated in regenerate tissues. We determined using our qRT-PCR screen that there is a significant increase in activin-βA mRNA but no significant upregulation of either TGFβ1, TGFβ2, TGFβ3 or BMP2 mRNA in early regenerate tissues. These findings indicate that activin-A, and not TGFβ1 or other TGFβ ligands, is likely the primary driver of SMAD2 phosphorylation during early stages of tail regeneration.

Previous studies on zebrafish fin regeneration have also reported that activin-βA mRNA is also the only TGFβ/activin ligand significantly upregulated in regenerate fin tissues (Jazwinska et al., 2007). This increase in activin-βA expression, combined with a conspicuous absence of TGFβ1, suggests that the type and levels of the TGFβ/activin isoforms expressed determines regeneration outcome and points towards activin-mediated signaling as an important mediator of both scar-free wound healing and the establishment (or maintenance) of cell proliferation during multi-tissue regeneration. In support of this, studies in chick granulosa cells demonstrated that activin-A treatment cooperates with protein kinase A (PKA) activity to cause a rapid, exclusive induction of SMAD2 mRNA and protein (Schmierer et al., 2003). This results in a shift in the SMAD2/SMAD3 ratio within a responding cell that preferentially increases SMAD2 phosphorylation and SMAD2 mediated transcription (Schmierer et al., 2003). Complementary to this, several studies have demonstrated that SMAD2 and SMAD3 target a subset of separate genes resulting in different transcriptional and cellular effects (Kretschmer et al.,
Further, SMAD2 knockout mice are embryonic lethal whereas SMAD3 mice are viable and following wounding, SMAD3 knockout mice show reduced ECM deposition, fewer fibroblasts and a smaller wound area when compared to control mice (Ashcroft et al., 1999). Taken together, these findings suggest that by selectively activating SMAD2, potentially through activin signaling, fibrotic responses can be attenuated. Further investigation of TGFβ/activin signaling during tissue regeneration, particularly on the function of activin’s in modulating the SMAD2/3 balance and subsequent transcriptional/cellular outcome, is necessary to establish activin’s role in scar-free wound healing.

**EMT transcription factors are upregulated during tail regeneration.**

The large pSMAD2 expression seen throughout regenerate tissues combined with previous work showing that when SMAD2 phosphorylation is blocked regeneration cannot proceed suggests that SMAD2 mediated transcriptional programs underly regenerate ability. TGFβ/activin mediated SMAD2 signaling can induce a variety of transcriptional effects dependant on cellular context, such as signalling pathway crosstalk (Guo and Wang, 2009). Based on the observations by Kragl and others that the rapid expansion of tissue specific stem cells, likely through dedifferentiation of adult cells, underlies regenerate ability we decided to examine TGFβ/activin induced epithelial to mesenchymal transition (EMT) transcription factors during tail regeneration. EMT transcription factors act to convert stationary epithelial cells into
a motile mesenchymal-like phenotype. During development, EMT serves as an essential mechanism guiding several phases of morphogenesis and organogenesis including gastrulation and neural crest delamination (Hay, 1995; Lim and Theiry, 2012). In adults, stimulation of the EMT program is known to promote organ fibrosis (Kalluri and Neilson, 2003) and tumor metastasis (Thiery, 2002). Recently however, it has been shown that EMT transcription factors can also induce a stem cell-like phenotype in both normal and transformed cell lines in vitro (Mani et al., 2008; Guo et al., 2012) and block cellular differentiation in vivo (Lai et al, 2012; Soleimani et al., 2012) potentially acting as cellular reprogramming agents.

We chose to examine the classical EMT transcription factors Snail1 and Snail2 which have both been implicated in stem cell induction as well as another EMT transcription factor Zeb2 (SIP1) that has been suggested to drive cell fate decisions (Lim and Thiery, 2012). Surprisingly, we observed a significant upregulation of mRNA encoding Snail1 and Snail2 in blastema tissue at stage IV of regeneration. To our knowledge, this is the first demonstration of EMT-associated zinc finger transcription factors Snail1 and Snail2 being upregulated during appendage regeneration and suggests that these transcription factors could be playing important roles in regeneration. Other studies using the axolotl have shown that the EMT associated basic helix loop helix (bHLH) transcription factors Twist1 and Twist3 are upregulated by blastema cells during both limb and tail regeneration (Satoh et al., 2008; Kragl et al, 2012). These findings suggest that EMT-associated transcription factors are involved in multi-tissue regeneration, however, the
function of these transcription factors in the regenerate program is yet to be
determined. It is tempting to speculate that Snail1 and Snail 2 are possibly
functioning in driving dedifferentiation that underlies the rapid expansion of the
regenerate blastema. Future work will need to extensively characterize the role of
these transcription factors during the regenerate program and are likely to yield
exciting results.
Summary and Conclusions

Numerous lizard species are capable of scar-free wound healing and subsequent multi-tissue regeneration following injury or loss of appendage (Tanaka and Reddien, 2011). An emerging model for the study of scar-free wound healing and regeneration in lizards is the leopard gecko (Whimster, 1978; McLean and Vickaryous, 2011; Delorme et al., 2012). Although the molecular regulation of the events that underly tail regeneration in the leopard gecko remain poorly understood, evidence from various other regeneration-competent models supports the involvement of various cytokines including members of the TGFβ superfamily. (Stoick-Cooper et al., 2007; Stocum and Cameron, 2011). In particular, the TGFβ/activin arm of the TGFβ superfamily has been shown to be essential for the regenerative response in other models of multi-tissue regeneration including zebrafish, Axolotl and Xenopus (Jazwinska et al., 2007; Levesque et al., 2007; Ho and Whitman, 2008). With this in mind we sought to investigate the TGFβ/activin signaling pathway as well as downstream TGFβ target genes at multiple stages during the leopard gecko regenerative program.

In this study we observed a robust induction of pSMAD2 throughout regenerate tissues from stages III to VI of tail regeneration. pSMAD2 was observed in the nuclei of numerous punitive blastema cells as evidenced by co-localization with the proliferation marker PCNA suggesting that blastema cells are actively responding to TGFβ/activin signals. This result confirms pervious work in other model systems that TGFβ/activin signals are highly active during multi-tissue regeneration.
To determine which TGFβ superfamily ligands were causing this robust induction of pSMAD2 throughout regeneration we performed immunohistochemistry and western blotting for the prototypical and profibrotic TGFβ ligand, TGFβ1. Interestingly we observed TGFβ1 expression is absent from early regenerate tissue but is observed in late regeneration as well as original tissue. This result indicates that TGFβ1 is likely not the main ligand responsible for the large pSMAD2 induction seen in regenerate tissue and suggests that an absence of TGFβ1 in early stages of tail regeneration may be necessary for successful scar-free, multi-tissue regeneration.

We then set out to detect the unknown pSMAD2 inducing ligand and interestingly observed that the only TGFβ/activin family member significantly upregulated in early regenerate tissues was *activin-βA*. Activin ligands are capable of causing SMAD2 phosphorylation and have been observed to be upregulated at early stages of zebrafish tail regeneration (Jazawinska et al., 2007). This results supports previous work in the zebrafish and suggests that activin ligands are playing unique and important roles in the tissue regeneration process.

We also observed that in early stages of tail regeneration epithelial to mesenchymal transition transcription factors are significantly upregulated in regenerate tissue. This suggests that the EMT is active during tail regeneration in the leopard gecko
and that EMT(s) could be an important, TGFβ mediated cellular programs that underlie regenerative ability.

These results support the previously described importance of TGFβ/activin signaling in numerous models of multi-tissue regeneration. Furthermore, the observation of dynamic TGFβ1 expression at various stages of regeneration combined with the observation of increased expression of activin-βA in regenerate tissues positions TGFβ/activin isoforms as important determinants of regenerate compliance and success. Finally, the upregulation of EMT transcription factors in early regenerate tissues suggests a model where specific TGFβ/activin isoforms can activate particular cellular programs, such as EMT, that contribute to regeneration and avoid scar formation. These TGFβ/activin signaling networks need to be further elucidated but likely hold much promise for improving our understanding of the factors that drive the balance between scar formation and tissue regeneration.
Significance and Future Directions

The results presented here improve our current understanding of TGFβ/activin signaling during scar-free wound healing and multi-tissue regeneration. The demonstration of robust TGFβ/activin signaling in the regenerate tissue of an amniote capable of multi-tissue regeneration is the first of its kind and expands on work demonstrating TGFβ/activin signaling in anamniotes capable of regeneration. Furthermore, this work alludes to the important roles of different TGFβ/activin ligands, in particular TGFβ1 and activin-βA during scar-free wound healing and multi-tissue regeneration. Work examining the protein expression of various TGFβ ligands in other models of multi-tissue regeneration has been lacking from the literature due to lack of appropriate antibodies. By examining protein localization through immunohistochemistry and western blot we were able to observe that TGFβ1 expression is absent from early regenerate tissue, a novel finding that is in line with other literature suggesting TGFβ1 is profibrotic and that its expression can drive scarification. Despite the absence of TGFβ1 ligand expression we still observe robust pSMAD2 nuclear localization in cells of the regenerate tissue, suggesting alternate ligands are driving SMAD2 phosphorylation. We identify that activin-βA is likely the pSMAD2 activating ligand in regenerate tissue. This result confirms the previously observed upregulation of activin-βA in zebrafish tail regeneration and highlights the potential importance of activin ligands during scar-free, multi-tissue regeneration. Finally we make the novel observation of EMT transcription factors Snail1 and Snail2 being upregulated in regenerate
tissue suggesting epithelial to mesenchymal transitions may be important for regenerative compliance.

To expand on this work more experiments should be conducted examining activin ligands, the localization of EMT transcription factors as well as gain and loss of function studies.

Activin ligands are formed from hetero and homo dimers with different inhibin subunits, thus, examining subunit mRNA expression with qRT-PCR does not reveal what ligands are assembled. These different ligands have been demonstrated to have different cellular effects and thus clarifying the dimer composition is an important next step for this project.

It will also be useful to better characterize the expression of EMT transcription factors Snail1 and Snail2 during tissue regeneation using techniques that localize their expression. EMT transcription factors are notoriously difficult to localize using antibodies and thus techniques such as in-situ hybridization may need to be considered. By localizing EMT transcription factors the identification of potential cells within particular tissues that may be undergoing epithelial to mesenchymal transitions can be determined. Once localized, further work can be conducted to examine what roles EMT transcription factors have in the tissue regeneration process.
Finally, gain and loss of function studies need to be conducted in-vivo and in-vitro to better understand the functional effects of TGFβ/activin ligands during the regenerative process in the gecko. With this in mind, useful techniques could be mRNA silencing using knockdown morphilinos and small RNA molecules, protein reduction using neutralizing antibodies, as well as pathway inhibition using small molecule inhibitors. Furthermore, the overexpression of different ligands could be accomplished through the implantation of agarose beads soaked in recombinant proteins, adenovirus mediated overexpression or through the treatment of tissue explants in-vitro. These different techniques could be applied to various research questions and would aid in our understanding of the functional consequences of under or over-expression of various TGFβ/activin ligands.
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Appendix I – Preparation of Materials

7.5% Acrylamide SDS-PAGE 2 X 1.5 mm Gels

Resolving Gel:
11 ml distilled water
3.72 ml 40% acrylamide-bis solution
5 ml Tris buffer (1.5 M, pH 8.8)
200 µl 10% SDS
10 µl TEMED
240 µl 10% APS (10 g APS dissolved in 100 ml distilled water)

10% Acrylamide SDS-PAGE 2 X 1.5 mm Gels

Resolving Gel:
9.7 ml distilled water
5 ml 40% acrylamide-bis solution 5 ml Tris buffer (1.5 M, pH 8.8) 200 µl 10% SDS
10 µl TEMED
240 µl 10% APS

12% Acrylamide SDS-PAGE 2 X 1.5 mm Gels

Resolving Gel:
8.7 ml distilled water
6 ml 40% acrylamide-bis solution
5 ml Tris buffer (1.5 M, pH 8.8)
200 µl 10% SDS
10 µl TEMED
240 µl 10% APS

Once resolving gel is polymerized, add the stacking gel: 3.2 ml distilled water
500 µl 40% acrylamide-bis solution
1.26 ml Tris buffer (0.5 M, pH 6.8)
50 µl 10% SDS
5 µl TEMED
50 µl 10% APS

Citrate Buffer

Stock Solution A (0.1M Citric Acid): 1.92 grams citric acid powder in 100ml deionized H₂O
Stock Solution B (0.1M Sodium Citrate dihydrate): 14.7 grams sodium citrate dihydrate powder in 500ml deionized H₂O

Mix 9.0 mL Solution A with 41 mL Solution B. Adjust pH to 6.0. Top up to 500mL with deionized H₂O, Store at 4°C

Electrophoresis Buffer
30.25 g Tris-base
144.1 g Glycine
100 ml 10% SDS
Dissolve in water to a volume of ~800 ml. Adjust pH to 8.3. Adjust final volume to 1 L. Store at room temperature

**IF Buffer**
0.1% BSA
0.2% Triton-X
0.05% Tween-20
Dissolved in PBS. Store at 4°C.

**8X Protein Loading Buffer**
400 mM Tris-HCl (pH 6.8) 16% SDS
0.8% Bromophenol blue 40% Glycerol
0.4 M DTT
Store at -20, once thawed, store in the dark at RT

**Semi-dry Transfer Buffer (1L)**
5.82g Tris
2.93g Glycine
200 mL Methanol
Dissolve in water to a final volume of 1L. Store at RT.

**TBS (10X)**
24.2 g Tris-base
80 g NaCl
Dissolve in water to a volume of 800 ml. Adjust pH to 7.6. Adjust final volume to 1L. Store at room temperature.

**Towbin Solution**
30.25 g Tris-base
144.1 g Glycine
Dissolve in water to a final volume of 1L. Store at 4°C.

**Transfer Buffer**
100 ml Towbin solution
200 ml Methanol
2.5 ml 10% SDS
Adjust volume to 1L with water. Chill at 4°C and use immediately.
Appendix II – Chemical List and Suppliers

Aurum Total Isolation RNA kit
Acrylamide/Bis solution (40%)
Alexa Fluor donkey anti-rabbit 488 IgG
α-Tubulin mouse mAb (B-5-1-2)
Aprotinin
APS
Bromophenol blue
BSA
Cy3 Goat anti-Mouse
DAB Peroxidase Substrate Kit
DC protein assay
DTT
GFX PCR Gel Band Purification Kit
Glycerol
Glycine
Goat anti-rabbit Biotinylated
Goat anti-mouse HRP
Goat anti-rabbit HRP
Na$_3$VO$_4$
PBS
PCNA mouse mAb (PC-10)
Phosphatase Inhibitor Cocktail 2
Phospho-Smad2 rabbit pAb (#3101)
PMSF
Reblot Plus (Mild)
Smad2 mouse mAb (#3103)
SDS
Sybr Green I
TEMED
TGFβ1 (V) rabbit pAb (sc-146)
Tris-base
Tris buffer (1.5 M)
Tris buffer (0.5 M)
Triton-X
Tween-20
Vectastain Elite ABC (Universal) R.T.U

Bio-Rad, Hercules, CA
Bio-Rad, Hercules, CA
Invitrogen, Camarillo, CA
Sigma-Aldrich, St. Louis, MO
Sigma-Aldrich, St. Louis, MO
Bio-Rad, Hercules, CA
Sigma-Aldrich, St. Louis, MO
Santa Cruz Bio, Santa Cruz, CA
Jackson Labs, West Grove, PA
Vector Labs, Burlingame, CA
Bio-Rad, Hercules, CA
Fisher Scientific, Nepean, ON
GE Healthcare, Cooksville, ON
Fisher Scientific, Nepean, ON
Fisher Scientific, Nepean, ON
Vector Labs, Burlingame, CA
Sigma-Aldrich, St. Louis, MO
Sigma-Aldrich, St. Louis, MO
New England Biolabs, Ipswich, MA
Lonza, Walkersville, MD
Dako, Burlington, ON
Sigma-Aldrich, St. Louis, MO
Cell Signaling, Danvers, MA
Sigma-Aldrich, St. Louis, MO
Millipore, Billerica, MA
Cell Signaling, Danvers, MA
Fisher Scientific, Nepean, ON
Applied Biosystems
Sigma-Aldrich, St. Louis, MO
Santa Cruz Bio, Santa Cruz, CA
Fisher Scientific, Nepean, ON
Bio-Rad, Hercules, CA
Bio-Rad, Hercules, CA
Bio-Rad, Hercules, CA
Fisher Scientific, Nepean, ON
Vector Labs, Burlingame, CA
Appendix III – Primers and Cloned Sequence Analysis

Table 1 Primers used for qRT-PCR

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<thead>
<tr>
<th>Primer</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>TGFβ1</td>
<td>5-TCGAGTCATGACAGCTACGG-3</td>
<td>5-GGTACAGCAGGTCCGGATTA-3</td>
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<td>TGFβ2</td>
<td>5-CACAATCCAGCCTATTTT-3</td>
<td>5-TGGCGAAGTTAGGCTTTTGC-3</td>
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<td>TGFβ3</td>
<td>5-AACGCATTGAGCTTTTCCAG-3</td>
<td>5-CGCACGGTATCTGTGACATC-3</td>
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<td>5-CCTGGCACATCTTTCCAGTT-3</td>
<td>5-GCTGGCTCAGTTTCTTGAC-3</td>
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<tr>
<td>Snail1</td>
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<td>5-CGGCTACCACATCTATGAA-3</td>
<td>5-TGGAGCTGAATTACCACGG-3</td>
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### Table 2 Primers used for cDNA identification

<table>
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<tr>
<th>Primer</th>
<th>Forward Primer</th>
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<td>5-AGGACAAAAGTGACCATCCG-3</td>
<td>5-GCTGGCTCCAGTTTCTTGAC-3</td>
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<td>5-CGGCTACCACATCCTATGAA-3</td>
<td>5-TGGAGCTGGAAATTACC CGCG-3</td>
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### Table 3 cDNA sequence analysis

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<th>Gene</th>
<th>cDNA (bp)</th>
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<th>Gallus gallus</th>
<th>Homo Sapiens</th>
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<td>99</td>
<td>99</td>
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</tbody>
</table>

*Activin βA was identified and published during the production of this manuscript by another group.

** Currently submitted to GenBank/NCBI awaiting accession numbers
Appendix IV – Cloned sequences

Activin βA

GGTAGTGTTGATAACTGTGGAGTGGAAAGAAAGGGTTGATCCAGATGTCCCCGCAATA
TGTTGGGGGAGCTCTCCTCACAGTACTGGATGGAGATCGATTGCATTTTGGATGGGAGATC
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ACACTCTTCTCTCTCTCTCTAGTCTCTCTCTCTTCTCTTCTCTTACCCGCAACAC
CAAGCTGGCTACATTTGATCAGAATACCAGACATCCCGAAGTGGAAAGTGGCAGGTACTTT
TCCTGGTACGAGACCTGGAATCTTCTTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
CCTCATCCCTCCACTNTCTTCTTCCATTTGGGAATGGGCACTCTCGTGGTACTG
TGFGβ1

CCAGATTCCTTAGTAAGCTCAAGCTGTCAGTTCCAGTCGGCCNGAAAGTGGGAAGAGGCCGCGACTT
TGCTGCTGAGTAGTTGGCACTTCAAACANGGATTTTAGTCAAGAAGAGGTC
ANGAGGAGCCAGAGACCCCAAGGAAGAAATATTACCCAAAGGCTGTTTACATGTT
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TGFGβ2

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TGF\(\beta\)3

TTCTGTACGCAGCTTCTTCTCCAGGTTTCGA AAACAGTAAATTTGAGTGCTAGTGCTCGTT TTTCTCTTGGCTTCTTACAGCTGGGCTCCAAGCCTATGTGGGGGAGCAT CATCAAAA TCAAGTGGGGAATTGGAAGGTCTTTCTCTTCTCTAGGCTCCTAAGTCCCCACGCTCAT GGCTACTCTCAG GCTGCATTGGAACTTTGAGTCTCAGCATGCTGCATTGCTCCAGGAGTTCTCCA AGATGTACACCATTGCTGACTGCTAGTATTACAT GGGCAGTGGATGCTGATCTCCAGTCCAGTCCC TTGGTAGATTCTCTATGCAAAAGCCATTCGGCAGGTTGACATCAA GAAGAAGGCAGGAACCCCGGTCATCACTCGTGGCGGAGCTAGGTAACCGCTGCTTTGCAAT ATGCTAGTGCGCTTCAAAAGCTTCAATGCTGCTGCAGTGGTGGGAAC TGGGTTTGGCATCGACAGCACAGGAAATTCAAGCC CAGGAAAGGGTGTGCTGCTGCTTCTT TCTGTGAAGACAGCTTGAAGGGGAAACAGTGGAGTTACCCCTTGGCGGAA TGT CAACCTATTGTTTGCTCAGGGGAGCTGTTTGGATCTCCTGGCTTCTGGTGAAGCTCCCGGGTGCTGTTGTAAAACGCAATC 18s

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