

SPATIAL EXPRESSION OF MATRIX AND MATRIX RECEPTOR PROTEINS IN THE DEVELOPING SYNOVIAL JOINT

By

J. Eli Robins

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Director: Dr. Anthony Capehart

Major Department: Biology

Little is understood about the complex spatial and temporal molecular interactions necessary to form a fully functional synovial joint. Understanding molecular pathways and signaling within the developing joint continues to be an area of constant discovery. Versican is a molecule with strong expression in the extracellular matrix (ECM) that has proven necessary for proper joint development.

The current study set out to discover gene expression patterns of ECM genes affected by the manipulation of versican. Five genes were chosen based on fold change differences identified from the results of an in-house 2010 microarray study. The microarray study evaluated gene expression differences when the G1 domain of versican was up-regulated in developing joint tissues using a recombinant adenoviral vector, and when the gene was silenced using adeno-shRNA. The five genes of interest were thrombospondin-3, discoidin domain receptor-2, sushi domain containing 5, matrix remodeling associated 5 and matrilin-4. *In situ* hybridization was performed on sagittal wing sections of HH35 (e9) chick embryos in an attempt to localize gene expression within the developing elbow joint.

Results demonstrate gene expression was present in the developing elbow region for each of the five genes of interest. Furthermore mRNA localization for each of the five genes extended

into surrounding tissues including the ectoderm, dermis, skeletal muscle, perichondrium, and chondrocytes. This study was undertaken to provide further insight into potential downstream effects of versican activity within the developing joint, as well as provide additional information on the presently limited knowledge surrounding the 5 genes of interest and their role in joint development.

SPATIAL EXPRESSION OF MATRIX AND MATRIX RECEPTOR PROTEINS IN THE
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By

Joshua Eli Robins

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Joshua Eli Robins

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By

Joshua Eli Robins

APPROVED BY:

DIRECTOR OF THESIS: _____

Anthony Capehart, Ph.D

COMMITTEE MEMBER: _____

Jean-Luc Scemama, Ph.D

COMMITTEE MEMBER: _____

Edmund Stellwag, Ph.D

COMMITTEE MEMBER: _____

John Wiley, Ph.D

CHAIR OF THE DEPARTMENT OF BIOLOGY: _____

Jeff McKinnon, Ph.D

DEAN OF THE GRADUATE SCHOOL: _____

Paul Gemperline, Ph.D

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TABLE OF CONTENTS

LIST OF TABLES.....	ix
LIST OF FIGURES.....	x
INTRODUCTION.....	1
Limb Development.....	1
Joint Formation.....	5
Versican Structure and Function.....	8
Genes Under Investigation.....	14
METHODS.....	20
Primer development and RT-PCR.....	20
Ligation and Transformation.....	21
Probe Generation.....	22
<i>In situ</i> Hybridization.....	23
RESULTS.....	26
Thrombospondin-3 (THBS3) Expression.....	26
Discoidin Domain Receptor 2 (DDR2) Expression.....	27
Sushi Domain Containing 5 (SUSD5) Expression.....	28
Matrix Remodeling-Associated 5 (MXRA5) Expression.....	29
Matrilin 4 (MATN4) Expression.....	30
Summary.....	30
DISCUSSION.....	58
Thrombospondin-3 (THBS3) Expression Pattern and Implications	

For Joint Development.....	59
Discoidin Domain Receptor 2 (DDR2) Expression Pattern and Implications	
For Joint Development.....	61
Sushi Domain Containing 5 (SUSD5) Expression Pattern and Implications	
For Joint Development.....	62
Matrix Remodeling-Associated 5 (MXRA5) Expression Pattern and	
Implications For Joint Development.....	64
Matrilin 4 (MATN4) Expression Pattern and Implications For Joint	
Development.....	64
Summary.....	66
REFERENCES.....	67
APPENDIX A: Animal Use Protocol.....	76
APPENDIX A: Discoidin Domain Receptor- 2 (DDR2) <i>in situ</i> hybridization in	
sagittal sections of chick wing at HH34 (e8).....	77
APPENDIX B: DDR2 HH34 (e8) areas of interest under increasing magnification.....	79
APPENDIX C: DDR2 <i>in situ</i> hybridization in sagittal chick Embryo HH25 (e5.5).....	81
APPENDIX D: DDR2 HH25 (e5) areas of interest under increasing	
magnification.....	83
APPENDIX E: SUSD5 <i>in situ</i> hybridization in transverse sections of Notochord	
at HH25 (e5.5).....	85

LIST OF TABLES

Table 1: Fold changes and accession numbers for versican affected genes from the 2010 microarray study.....	13
Table 2: Genes, accession numbers, primer sequences and probe lengths for <i>in situ</i> hybridization.....	32
Table 3: Nano-drop quantitation of purified plasmids.....	36
Table 4: Summary of mRNA expression for each gene categorized by tissue.....	57

LIST OF FIGURES

Figure 1: Growth pathways during limb development.....	4
Figure 2: The four isoforms of versican formed by alternative splicing.....	12
Figure 3: Gel electrophoresis data confirms approximate insert size.....	33
Figure 4: Confirmation of cloned insert sequences based on DNA sequencing using T7 sequencing primers.....	34
Figure 5: Thrombospondin-3 (THBS3) <i>in situ</i> hybridization in serial sagittal sections of chick wing at HH35 (e9).....	37
Figure 6: THBS3 areas of interest under increasing magnification.....	39
Figure 7: Discoidin Domain Receptor-2 (DDR2) <i>in situ</i> hybridization in serial sagittal sections of chick wing at HH35(e9).....	41
Figure 8: DDR2 areas of interest under increasing magnification.....	43
Figure 9: Sushi Domain Containing 5 (SUSD5) <i>in situ</i> hybridization in sagittal sections of chick wing at HH35(e9).....	45
Figure 10: SUSD5 areas of interest under increased magnification.....	47
Figure 11: Matrix Remodeling-Associated 5 (MXRA5) <i>in situ</i> hybridization in serial sagittal sections of chick wing at HH35(e9).....	49
Figure 12: MXRA5 areas of interest under increased magnification.....	51
Figure 13: Matrilin-4 (MATN4) <i>in situ</i> hybridization in serial sagittal sections of chick wing at HH35 (e9).....	53
Figure 14: MATN4 areas of interest under increased magnification.....	55

1. INTRODUCTION

Limb Development

Limb development begins with the formation of the limb buds, which sprout from the lateral plate mesoderm. This occurs when mesenchymal cells initially secrete fibroblast growth factor 10 (Fgf10). Wnt2b and Wnt8c signaling localize and stabilize Fgf10 expression to the respective fore- and hind limb bud region (Ohuchi et al. 1997, Kawakami et al. 2001).

As limb buds begin to expand from the recruitment of mesenchymal cells, their continued secretion of Fgf10 creates an ectodermal covering on the distal tip of the limb bud called the apical ectodermal ridge (AER) (Gilbert 2006). This major cellular signaling center is critical and necessary for limb bud growth and development (Saunders 1972; Krabbenhoft and Fallon, 1989).

Limb growth can be described along three axes: The proximal-distal axis describes growth from the shoulder to the fingertips; The anterior-posterior axis defines growth from thumb to little finger; Lastly dorsal-ventral growth differentiates the palm from the back of the hand. While examined individually, all growth axes are interrelated in regards to their signaling pathways (Gilbert 2006).

Proximal- distal growth of the limb bud extends the limb toward the fingertip. It is facilitated by a positive feedback loop involving fibroblast growth factors found in the AER and the Progress Zone (PZ) (Gilbert 2006). The progress zone is immediately proximal to the AER, and is composed of undifferentiated mesenchymal cells that constantly proliferate and secrete Fgf10. Fgf10's expression creates and continually maintains the AER (Xu et al. 1998). The AER also mediates the synthesis of Fgf8, which is responsible for maintaining mesenchyme in a relatively undifferentiated state and facilitating continued proliferation of cells in the PZ. This creates a continual feedback loop promoting limb bud elongation.

Anterior-posterior growth specifies digit identity from the second to fourth phalange. Signaling from the Zone of Polarizing Activity (ZPA) and AER mediate this patterning (Gilbert 2006). The ZPA is found proximal to the AER and expresses sonic hedgehog (Shh) (Riddle et al. 1993). Shh is indirectly induced by Fgf8 from the AER. When Fgf8 is secreted into the ZPA, transcription factors Hoxb8 and dHAND, regulate the activation of Shh (Charite et al. 2000). Shh and Fgfs work in a positive feedback loop controlling each other's activity. Shh activates Fgf4 in the posterior portion of the AER where it assists with recruitment of mesenchyme cells to the PZ. Working in conjunction with Fgf8, Fgf4 regulates Shh's expression in the ZPA (Gilbert, 2006). Shh further protects the Fgf's in the AER by activating Gremlin, an inhibitor of bone morphogenic proteins (BMP's) (Zuniga et al. 1999). BMPs inhibit the AER by blocking FGF expression.

Growth along the dorsal-ventral axis distinguishes the palmar surface from the knuckle side. The polarity is dependent on Wnt7a. Expressed within the dorsal ectoderm, its expression induces Lmx-1, which provides further specificity to dorsal mesenchyme (Dealy 1993; Parr et al. 1993). Its deletion however leads to ventral pads on the dorsal side (Gilbert 2006). Wnt7A is also involved in anterior-posterior, and proximal-distal patterning. Wnt signal suppression knocks down Shh expression causing a loss of posterior digits (Yang and Niswander, 1995). By contrast overactive Wnt7a signaling causes an extensive growth in the AER leading to an excess of digits (Adamska et al. 2004).

Later stages of limb development are also controlled through Wnt signaling in non-canonical pathways. One non-canonical pathway, the planar cell polarity pathway (PCP), is involved in modulating cellular structure. The PCP pathway is implicated in structural management of the actin cytoskeleton (Komiya et al. 2008). This involves maintaining the

proper orientation of growth plate chondrocytes for continuous lengthening and differentiation (Li and Dudley, 2009). The interruption of this pathway results in structural defects in cartilage orientation, causing defects in the synovial joint (Li and Dudley, 2009).

Cessation of limb outgrowth occurs from spatial distance between differentiated structures and protein interactions. Spatially, the positive feedback loop established by the ZPA and AER, inhibits itself by creating a barrier. The influx of ZPA cells over time increases the spatial distance between structures blocking Shh signaling to induce gremlin (Gilbert 2006). The inactivation of gremlin activates BMPs, which inhibit the continued activity of the AER (Scherz et al. 2004). Furthermore BMPs inhibit Wnt7a expression along the dorsal-ventral axis (Gilbert 2006).

Figure 1. Growth pathways during limb development

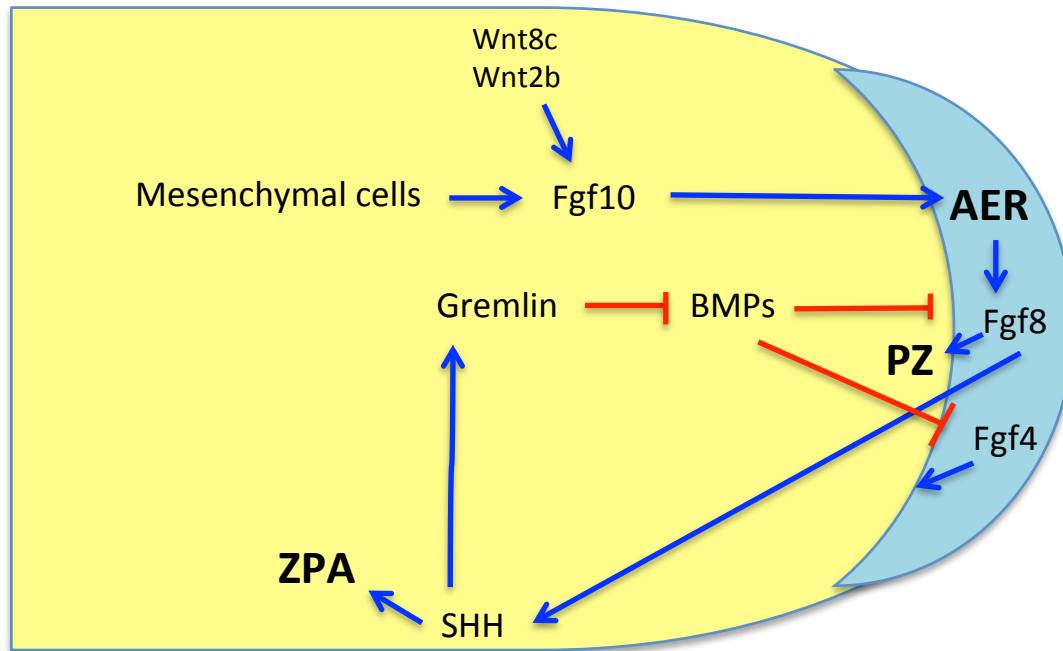


Figure 1. Illustration of the pathways responsible for the regulation of the three interrelated axes of limb growth and development. Beginning with mesenchymal cells, blue arrows represent activation of various downstream targets, while red lines indicate inhibition.

Joint Formation

Synovial joint formation in developing organisms remains, in large part, poorly understood. Deciphering the complex molecular signaling pathways that give rise to multiple tissues in specific temporal and spatial arrangements, is needed to fully define the developing joint. While there are still a plethora of questions, the basic mechanisms of joint formation beginning with prechondrogenic mesenchymal cells have been delineated.

Interzone formation is the first observable sign of joint development and demarcates the eventual site of the presumptive joint (Holder, 1977). The formation of the interzone is characterized by the aggregation of densely packed mesenchymal cells (Pacifici et al. 2005). Experiments have proven its removal leads to the absence of the joint (Pacifici et al. 2005).

A large area of research has been focused on gene expression patterns and signaling pathways within the interzone. The canonical Wnt/ β -catenin pathway has proven to be both necessary and sufficient for joint formation. Wnt 4, Wnt9a, and Wnt16 are expressed during early joint formation and may possibly play a role in the early expression of β -catenin. When a Wnt ligand binds to its Frizzled receptor, the recruitment of Disheveled causes the subsequent inactivation of GSK3. This allows β -catenin to translocate into the nucleus where it acts as a transcription factor known to activate several genes necessary for joint development (Liu et al. 2005). Some of downstream effectors include CD44, GDF5, chordin, and autotaxin (Hartmann and Tabin, 2001). When β -catenin is ablated, joint formation does not occur (Guo et al. 2004).

The formation of the interzone involves inhibition and activation mediated by the Wnt pathway. A downstream target inhibited by the β -catenin expression is Sox9. Involved in the activation of type IIA collagen, its inhibition arrests chondrocyte differentiation allowing for the induction of joint formation (Bell 1997, Ng 1997). The activation of Growth/Differentiation

Factor 5 (GDF5) promotes chondrogenesis and chondrocyte proliferation (Storm and Kingsley, 1996). As joint formation begins, its expression in the interzone is believed to promote further signaling involved in maintenance and development of the joint (Storm and Kingsley, 1999).

BMPs, which later arrest growth of the limb, as well as their antagonists such as Noggin, also play a role in joint development once the interzone is established (Francis-West et al. 1994). Noggin regulates proliferation of the interzone to ensure normal progression of events during joint formation (Pacifici et al. 2005).

Once the interzone is established, and mesenchymal cells aggregate in response to Wnt/ β -catenin signaling, cavitation occurs. This encompasses the physical separation of the mesenchyme, which creates the presumptive synovial cavity. The activities of hyaluronin (HA) and CD44 are integral in creating this morphological change (Craig et al. 1990). HA, an unsulfated glycosaminoglycan, and CD44 are widely expressed within the interzone (Archer et al. 1994). Interaction between HA and CD44 can promote both tissue adhesion and loss of tissue integrity (Toole, 1991). As HA synthesis increases beyond CD44 receptor saturation, tissue integrity is lost within the intermediate area of the interzone (Pacifici et al. 2005). Inactivation of HA in the developing limb has proven to cause severe abnormalities in both limb growth and joint formation (Matsumoto et al. 2009). It's absence results in improper cavitation, digit formation, and delayed hypertrophy within the limb. Cell death is not believed to play a significant role in cavitation, rather changes in matrix structure are thought to accommodate and promote further cellular spacing during cavitation (Pacifici et al. 2005).

Following cavitation, morphogenesis creates complementary articular connections forming the complete synovial joint. This area remains the least understood due to the plethora of molecular interactions involving various tissue types and physical cues. One molecule that

continues to be expressed throughout joint development is GDF-5 (Pacifci et al. 2005). Known to drive chondrogenic growth and differentiation, it is believed to be involved in creating the complementary connection between the proximal and distal ends of the joint (Francis-West et al. 1999).

One physical cue that plays a role in morphogenesis is the myogenic strain placed on the articulating bones from forming muscles. This variable along with the multitude of signaling pathways has made post-cavitation studies very difficult. Laboratory manipulations *ex vivo* have been unsuccessful in forming a functional synovial joint (Holder, 1977). While a presumptive joint area forms, cavitation does not occur (Archer et al. 1994). The inability to create functional joints *in vitro* has limited the methods used to study molecular signaling pathways.

Versican

Versican is a chondroitin sulfate proteoglycan, which is highly expressed in the extracellular matrix of the developing chick and mouse joint (Shepard et al. 2007; Snow et al. 2005). During the initial induction of joint formation, its expression is found within the perichondrium and through out the mesenchymal cells that form the interzone (Shepard et al. 2007). As morphogenesis continues, its localization becomes restricted to the articular cartilages of the respective bones forming the synovial joint. Versican's role in chondrogenesis has yet to be fully delineated, but it is known to be involved in cell adhesion, proliferation, and migration (Rahmani et al. 2006). It also appears to be involved in the requisite step of mesenchymal aggregation leading to expression of the chondrocyte phenotype (Williams et al. 2005; Shepard et al. 2008).

The core protein of versican consists of a glycoprotein covalently attached to glycosaminoglycans (GAGs). The chondroitin sulfate (CS) complement defines the disaccharide structure of the GAG attached to the glycoprotein. Versican is a member of the hyalectin family, a group of chondroitin sulfate proteoglycans that bind hyaluronan. Versican however, is the most widespread with regard to expression and distribution throughout the body (Kinsella et al. 2004). Its functional versatility is achieved by having 3 binding domains and 4 active isoforms.

The three functional domains of versican are G1, G2, and G3 (Wright, 2002). The G1 domain is found at the N-terminus of the molecule and is responsible for its interaction with hyaluronan. The G2 domain encompasses the chondroitin sulfate GAG side chains, GAG α and GAG β . The negative charge of the CS chains facilitates binding to a number of cellular signals, and it is believed to be responsible for versican's anti-adhesive properties (Yamagata and Kimata, 1994; Sakko et al., 2003). Lastly the G3 domain, found on the C-terminus, consists of

two ectodermal growth factor repeats (EGFs), a C-type lectin domain and a complementary regulatory region. The numerous binding sites give the G3 domain substantial functional versatility in its interactions. It has proven to have adhesive properties, binding integrins to activate focal adhesion kinase (FAK) (Wright, 2002), as well as involvement in proliferation and migration, through its EGF repeats (Zhang et al. 1998). Alternative splicing of versican creates 4 active isoforms. V0 contains all functional elements of the CS proteoglycan. V1 and V2 differ in the CS GAG chain they contain. The V2 isoform contains the GAG α domain, while V1 contains GAG β . The final isoform V3 lacks both GAG α and GAG β (Shinomura et al. 1993).

The four isoforms of versican can be found in a variety of tissues throughout the body. Of the four isoforms, V0/V1 predominate in the developing limb (Snow et al. 2005), and V3 mRNA has been localized in the limb core mesenchyme (Hudson et al. 2010). Versican's G1 domain is of particular interest due to its interaction with hyaluronan. Hyaluronan, which is believed to be a major contributor of joint cavitation, binds to its cell surface receptor CD44. This induces proliferation as well as cellular migration (Pacifici et al 2005). Furthermore *in vitro* studies have shown hyaluronan's interaction with versican enhances proliferation and reduces integrity of tissue structure between cells (Zhang et al. 1998). Versican's role in modulating the interaction of hyaluronan in the presence of CD44 has yet to be determined. When versican was conditionally knocked out in mice however, joint development resulted in limb deformities, tilting of the joints, and improper cavitation within the interzone (Choocheep et al. 2010). Suppression of versican in the developing chick joint also demonstrated defects in interzone structure (Nagchowdhuri et al. 2012).

Along with the effects of hyaluronan, Wnt signaling has proven to be a significant contributor in limb and joint development (Gou et al. 2004). Wnt signaling activates the PI3K/

PKB pathway, which inhibits the expression of GSK-3 β . This allows for the accumulation of β -catenin and its translocation to the nucleus. There, it activates several target genes including versican's promoter (Rahmani et al. 2005). Other genes activated by β -catenin include fibronectin, cyclinD1, matrix metalloproteinase (MMP)-7 and c-myc (Goodwin and D'Amore, 2002). While Wnt signaling modulates versican's expression, potential downstream effects through non-canonical pathways have yet to be fully delineated.

In previous studies, individual domains of versican have shown their ability to function independently of the intact proteoglycan (Kern et al. 2006, Kern et al. 2007, McCulloch et al. 2009). The G1 domain of versican has been implicated in a variety of cellular processes, both complementary and inhibitory when bound to HA (Zhang et al. 1998; Yang et al. 1999). Most recently, the G1 domain and V3 isoform proved to increase chondrogenic aggregation, resulting in enlargement of cartilage at sites along the humeral primordium (Hudson et al. 2010). Furthermore, its knockdown caused a decrease in other ECM molecules associated with the developing joint including collagen II, hyaluronan, link protein 1 and tenascin (Nagchowdhuri et al 2012). Moreover, a proteolytic fragment containing the G1 domain is generated during murine joint development, suggesting a function independent of full length versican (Capehart 2010). Versican proteolysis yielding the G1 fragment is thought to be mediated by ADAMSTS family members and matrix metalloproteases (Kern et al. 2007)

The Capehart lab generated microarray data that was recently compiled from two experimental groups: an up regulated versican G1 domain and an shRNA-induced versican knockdown. The overexpression of the G1 domain was performed through use of an adenoviral construct, and the knockdown was enabled through adenoviral encoded shRNA interference (Hudson et al. 2010; Nagchowdhuri et al. 2012). After microinjection of the two experimental

groups, RNA was extracted from the developing elbow joint at HH35 (e9), and microarray analysis was performed by the Genomics Core at the University of North Carolina at Chapel Hill.

The present study is focused on localization of transcripts from five matrix and matrix receptor genes within the developing chick elbow that exhibited changes in gene expression in response to G1 overexpression and versican knockdown (Table 1). The potential expression and role of these 5 genes in the developing chick joint are largely unknown at this time.

Figure 2: The four isoforms of Versican formed by alternative splicing.

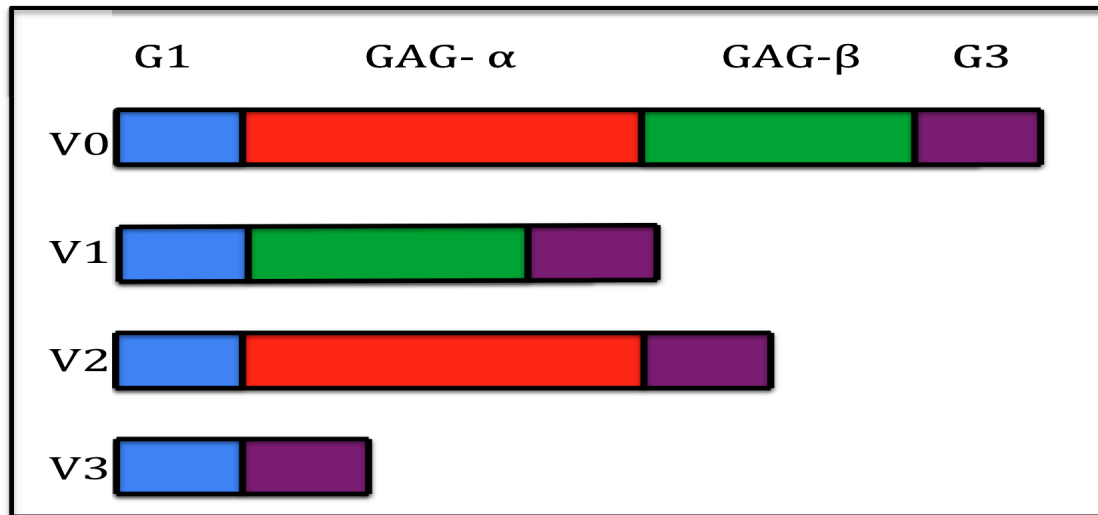


Figure 2. Schematic diagram representing the three functional domains of versican in each of its 4 isoforms. Blue represents the G1 domain, which has a HA binding region. Red and green represent the GAG- α and GAG- β domain of G2 respectively. The G3 domain is depicted in purple and contains two EGF regions, a lectin binding domain, and a complement regulatory domain involved in protein-protein interactions.

Table 1. Fold changes and accession numbers for versican affected genes from the 2010 microarray study

Genes	Accession Number	Fold Change
Thrombospondin 3 (THBS3)	L81165.1	2.4
Discoidin Domain Receptor-2 (DDR2)	XM_422211.3	2.2
Sushi Domain Containing 5 (SUSD5)	XM_418822.3	2.4
Matrix-Remodeling Associated 5 (MXRA5)	XM_416853.3	-2.2
Matrilin 4 (MATN4)	XM_425698.3	2.4

Table 1. List of versican affected genes followed by accession number and fold change statistics from the 2010 microarray experiments. Positive fold change describes over-expression due to adeno-G1 induced versican, while negative fold change indicates RNA knockdown via shRNA versican silencing.

Genes Under Investigation:

Thrombospondin-3 (THBS3)

Thrombospondin-3 is one of the five members in the Thrombospondin family. While its function is not yet fully defined, it has been expressed in a variety of tissues including osteocytes and chondrocytes during embryogenesis (Qabar et al. 1995). Like other family members THBS3 is comprised of calcium binding repeats, and epidermal growth factor (EGF) repeats. It is most similar to THBS4 and 5, but all are all capable of oligomerization. Furthermore all three contain 4 EGF2 repeats rather than 3 found in THBS1 and 2 (Hankenson et al. 2005). THBS3 is distinct at its N terminus showing no homology to other thrombospondins, however its function has yet to be uncovered. The protein also has heparin binding activity, which like THBS1, could lead to interactions with sulfated proteoglycans (Qabar et al. 1994).

Gene expression of THBS3 has been detected within the growth plate and the perichondrium of the developing murine limb *ex-utero* (Qabar et al. 1994). In the avian limb THBS3 mRNA was found localized to hypertrophic chondrocytes, as well as articular and proliferative cartilages during embryogenesis (Tucker et al. 1997). In earlier stages of development, THBS1 was found in similar locations, leading to the possibility of temporally complementary expression patterns in the THBS- family (Tucker et al. 1997).

THBS3 knockouts have yielded functional joints with phenotypic distortion specifically due to postnatally accelerated endochondrial ossification and trabeculation in the femoral head (Hankenson et al. 2005). Furthermore, chondrocytes showed a significant decrease in columnar alignment giving insight into THBS3's possible temporal and spatial role during limb growth *ex-utero* (Posey et al. 2008; Hankenson et al. 2005). Prenatal skeletal patterning was unaffected by THBS3 knockout.

Discoidin Domain Receptor-2 (DDR2)

Discoidin Domain Receptor 2 is a Receptor Tyrosine Kinase (RTK) that is activated by triple helical collagen (Zhang et al. 2011; Konitsiotis et al 2008). Mutations in the gene have proven to cause spondylo-meta-epiphyseal dysplasia [SMED], a severe deformity characterized by limb shortening and abnormal calcifications in humans (Bargal et al. 2009). Molecularly its knockout has been implicated in reduced chondrocyte and fibroblast differentiation (Labrodor et al. 2001). DDR2 is a non-integrin type receptor, which regulates cell adhesion, proliferation, and extracellular matrix (ECM) remodeling (Vogel et al. 2006). Distinguishing DDR2 from other RTKs is its discoidin domain. Loops 1 and 3 within this domain are integral for collagen binding and receptor activation (Abdulhussein et al. 2004). DDR2's maximum activation occurs over several hours and can last up to 16 hours. This is significantly longer when compared to other RTKs (Vogel et al. 2006). Post-activation, the molecule has 13 sites for possible tyrosine phosphorylation (Vogel et al. 2006).

While expressed in several different types of tissue, DDR2's involvement in limb development is thought to be in osteoblast differentiation and chondrocyte maturation. Mesenchymal stem cells undergo chondrogenic differentiation, creating a cartilaginous template, which later forms into bone (Karsenty, 2003). The expression of Collagen type 1, DDR2 and RUNX2 characterize this conversion (Franceschi et al. 2007). Collagen type 1 activates DDR2, which causes an increase in mRNA levels during the early stages of osteoblast differentiation (Lin et al. 2010). This corresponds with the transcriptional activity of RUNX2, which DDR2 has been shown to regulate (Zhang et al. 2011). Involved in chondrocyte hypertrophy, RUNX2's absence is embryonic lethal in homozygous mice, and shows severe structural abnormalities in heterozygous knockouts (Hartmann 2009; Mundlos et al. 1997).

Detailed studies of DDR2 suggest that it is involved in modulating several downstream pathways and proteins including p38/MapK, ERK, and MMP activity. Found in the p38/MapK pathway and known to play a role in osteoblast differentiation, activating transcription factor-4 (ATF4) is able to up-regulate DDR2 and RUNX2 expression when bound to DDR2's promoter region. The expression of extracellular signal-regulated kinase (ERK) has also been discovered during DDR2's modulation of RUNX2. ERK's responsiveness to DDR2 signaling has proven to rescue RUNX2 mutants inducing osteoblast and chondrocyte differentiation (Zhang et al 2011). Furthermore DDR2 has been found to influence the expression of various matrix metalloproteinases (MMPs), which are known to be active during joint and limb development (Olaso et al. 2002; Capehart 2010; Ikeda 2002). While DDR2 expression within the developing limb has been characterized, its expression during the early stages of joint development still remain unknown.

Sushi Domain Containing 5 (SUSD5)

KIAA0527 was recently renamed SUSD5 in humans. There is very little known about the molecule's expression or function. It has been identified in microarray studies in the developing elbow by the Capehart lab, as well as in the temporomandibular joint (Shibukawa et al. 2007). Structurally, the molecule contains a link domain and a sushi domain (Vincent 2008). The link domain encodes a proteoglycan tandem repeat domain (PTR) that interacts with hyaluronan (Brissett and Perkins 1996). Hyaluronan has proven to be highly expressed within the interzone interacting with CD44 to promote cavitation, suggesting that the hyaluronan-binding link domain of the SUSD5 protein may play a similar role. Furthermore, real time PCR has indicated that with G1 overexpression of versican, SUSD5 had a significant fold change in gene expression.

The significance of another hyaluronan receptor in addition to CD44 has yet to be investigated in the developing joint.

Matrix Remodeling-Associated 5 (MXRA5)

Matrix-Remodelling Associated 5 is the updated name for Adlican. It is an adhesion proteoglycan, able to bind vascular endothelial growth factor (VEGF) (Walker et al. 2002). The protein is composed of three leucine repeats found on the N-terminus and 12 immunoglobulin domains spanning throughout the molecule. Very little is known about the gene in terms of synovial joint development or in long bone development and growth. It is, however, implicated in extracellular matrix growth and remodeling (Donghai et al. 2012). Studies have shown increased gene expression in osteoarthritis, as well as in skin fibroblasts of centenarians (Karlsson et al. 2010; Chondrogianna et al. 2004). The gene is also up regulated in high-risk, radiation-induced fibrosis patients (Rodningen et al. 2008). Recent studies have labeled it a novel cancer gene due to the identification of mutations in this gene that correlate with the occurrence of non-small cell lung carcinomas (Xiong et al. 2012).

Matrilin-4 (Matn4)

Matrilin-4 is one of four members in the matrilin family. Initially labeled as cartilage matrix protein, the discovery of matrilins 2, 3, and 4 have lead to a new classification. Found predominantly within cartilage ECM, the matrilin family is thought to mediate interactions between collagens and ECM proteins (Hauser and Paulson 1994). Matrilin4 (Matn4) is the most recent member to be identified, and is the most universally expressed (Klatt et al. 2001).

Structurally this ECM protein does not vary significantly from other matrilins. In the mouse, MATN4 contains two Von Willebrand factor A (VWA) domains, connected by four ectodermal growth factor (EGF) domains (Wagener et al. 2005). MATN4 EGFs can range in

number depending on the organism and differential splicing (Wagener et al. 1998). Studies have examined matrilin EGF involvement within the non-canonical Wnt pathway, and concluded there is not a non-canonical calcium-binding site with the EGF domain (Malby et al. 2001). Matrilins also contain a C-terminal α -helical coiled-coil domain allowing for oligomerization. This can induce homo-trimers for MATN 2 and 4, and homo-tetramers for MATN1 and 3. Reasoning behind oligomerization is unknown, but it is thought to involve the vWFA domains and possibly affect cellular signaling (Hauser and Paulsson, 1994; Klatt et al. 2001).

The molecular similarity within the matrilin family allows members to compensate in another's absence (Wagener et al. 2005). MATN4 is unique, as it appears to be universally present where other matrilins are expressed (Klatt et al. 2001). Furthermore, it has also shown the ability to be proteolytically cleaved from its oligo-trimer form (Klatt et al. 2001). This could play a significant role in tissue signaling by modulating MATN4's interaction and binding with other ECM molecules (Klatt et al. 2001).

In embryonic mice, MATN4 is found in both dense and loose connective tissue. In dense tissue, the protein is present at the joint surface, as well as surrounding cartilage (Klatt et al. 2001). This includes the perichondrium, periosteum, proliferating, and hypertrophic cartilages (Klatt et al. 2001). Interestingly it is the only matrilin expressed on the surface of developing articular cartilage (Klatt et al. 2001).

In the chick model, MATN4 expression has been evaluated at various embryonic stages within the tibio-tarsus (Bandyopadhyay et al. 2008). Spatial localization was found in a graded expression pattern, with the strong signal subjacent to the perichondrium in HH35 (e9) chicks. The same study also concluded as development continued (stage HH34 to HH38), expression patterns shifted away from chondrocytes undergoing hypertrophy and toward the epiphyseal

ends of the developing long bones. One explanation is that MATN4 may be positively regulated by TGF β , which is produced and localized in the perichondrium. *In vitro* studies have proven TGF β increases intracellular MATN4 levels and inhibits chondrocytes from differentiating (Bandyopadhyay et al. 2008).

2. METHODS

Primer Development and RT-PCR

Complementary DNA (cDNA) constructs were developed through reverse transcription of WT HH35 limb RNA using SuperScript III First Strand Synthesis Kit (Invitrogen). Specific primers for the 5 genes under investigation were designed to verify transcript presence in HH35 (e9) joint tissues using *ncbi.nlm.nih.gov* resources, including nucleotide BLAST function, to confirm their specificity against the *Gallus gallus* genome. Transcript lengths ranged from 100-200 base pairs.

Reverse transcriptase PCR followed to verify gene expression within the synthesized cDNA. This was conducted using 25µl samples containing 15.125µl dH₂O, 2.5µl buffer, 0.75µl MgCl₂, 0.5µl dNTPs, and 0.125µl Taq polymerase, 1.0µl cDNA, 2.5µl forward and reverse primers (30mM). The reaction took place in an Eppendorf Master cycler, using an annealing temperature gradient specific to each primer. A total of 35 cycles were carried out, each having a 2:00 minute denaturation stage (94°C), a 30 second denaturation stage at (94°C), a 30 second annealing phase (primer dependent temperature), and a 90 second elongation phase (72°C). After 35 cycles a final elongation stage occurred lasting 10 minutes (72°C). The cycler then held the product at 15°C.

DNA products were analyzed using gel electrophoresis. The 1.5% agarose gels were prepared using 50mL 1X TAE buffer and 0.75g of agarose. Five microliters of 1% ethidium bromide was added once the mixture cooled enabling the products to be visible under UV light. DNA products were diluted 4:1 using DNA loading dye (Invitrogen) and gels were electrophoresed for 60 minutes at 85 volts. Gels were photographed under UV light and successful primers were held for possible *in situ* primers.

In-situ primers were designed and analyzed using the same methods however the product length was increased, ranging 450 to 550 base pairs. Once all DNA products were verified using gel electrophoresis, 50µl PCR reactions were performed to amplify the products. The reagents involved in the reaction were doubled respectively. DNA was excised from the gel post-gel electrophoresis, and purified from agarose using a QIAquick Gel Extraction Kit (Qiagen).

Ligation and Transformation

Once purified, the amplicons were ligated into the pGEM-T Easy vector (Promega) using 5µl 2x ligation buffer, 1µl pGEM-T Easy vector, 3µl PCR product, and 1µl T4 DNA ligase. The reaction incubated overnight at 15°C.

The ligation product was then transformed into JM109 cells. 50µl of JM109 cells were added to 2µl of the ligation reaction. Cells were heat-shocked for 50 seconds at 42°C. 950µL of SOC medium was then added and cells were placed in a shaker to incubate 1.5 hours at 37°C at 225 RPM. After incubation, cells were spun into a pellet and plated onto LB-Ampicillin media, which contained 100µl of 100mM IPTG and 20µl of 50mg/mL X-gal. White colonies were chosen after growth occurred on the plate, and small cultures were made utilizing 5mL LB and 50 mg/ml Ampicillin. Cells were shaken overnight at 37°C at 225 RPM.

Following incubation, the cells were harvested at 4000 RPM for 3 minutes (IEC Centra CL2) and plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen). Three microliter samples of the purified plasmids were used in an EcoR1 digest, followed by gel electrophoresis to confirm the presence of the expected insert. The gel protocol paralleled that used for DNA product identification. Following purification, a nanodrop (Thermo Fisher) was used to determine plasmid concentrations. Plasmid DNA was sequenced using the Genomic Core Facility. The cloned insert sequences were compared to the genomic sequence from *Gallus gallus*

in NCBI using the BLAST algorithm. The results from BLAST searches were used to confirm insert identity, and specificity. Analysis also gave insight into the insert's orientation within the plasmid.

Probe generation and staining

Probe generation began with linearizing THBS3, DDR2, SUSD5, MXRA5, and MATN4 plasmids with Nco1 or Nde1. Each digestion contained 20 μ l (4000 ng) of purified plasmid, 5 μ l of buffer, 11.5 μ l of water, 3 μ l of enzyme (Nco1 or Nde1), and 0.5 μ l of 10 mg/ μ l BSA. The digests were incubated overnight at 37 °C. The following day gel electrophoresis was performed under the same conditions as earlier stated. The linearized plasmids were excised from the gel using a RNase/DNase free sterile razor and purified using a Qiagen Plasmid Mini Kit. The probe reaction followed. The probe reaction is most efficient when linearized plasmid concentration is above 1000 ng/ml. Roche protocol was used utilizing 14 μ l (1000 ng) linearized plasmid, 2 μ l 10x transcription buffer, 2 μ l DIG label + nucleotides, and 2 μ l of T7 or SP6 polymerase depending on the digest. Nco1 digests utilize SP6 and Nde1, T7 polymerase. The reaction was performed for 2 hours at 37°C.

Probe activity was assessed by dot blot. The dot blot used 1 μ l of sample blotted onto a nylon membrane. Subsequent blotting used a 2X serial dilution of the probe creating a gradient along the nylon membrane, and was crosslinked covalently to nylon using a cross linker (Stratagene). The membrane was submerged 15 minutes in 4ml 1x block at 37°C (Roche), and incubated in anti-DIG 1:2000 for 30min. The membrane was washed using TBS with 3% Tween 10 minutes, and TBS 10 minutes, before development with a NBT/BCIP tablet (Roche). Probe concentrations were measured subjectively by comparing colorimetric detection produced from anti-DIG's interaction with the NBT/BCIP substrate to the known concentrations of the DIG

labeled control. All probes were standardized to the intensity exhibited by 0.1 ng/ml of DIG labeled control. This gave a consistent level of probe activity for each probe for beginning *in situ* hybridization.

***In situ* Hybridization**

In situ hybridization was performed over two days modified from Brietschopf et al. (1992). Previously dissected wild-type left chick wings (AUP in Appendix A) at HH35 (e9) were fixed and stored in 85% ethanol, 10% formaldehyde, and 5% glacial acetic acid at 4°C. Fixed tissues were embedded for histological sectioning according to Hudson et al. (2010). Sagittal sectioning of the wing was performed at 11 microns. Sections were mounted at intervals of 22 microns enabling experimental and control slides to contain sequential sections. The method for *in situ* hybridization was performed under RNase/ DNase free conditions using 0.01% DEPC treated water. Sections selected for in situ hybridization were chosen based on tissue integrity and elbow joint illustration. Each experimental slide was used with the corresponding serial control slide. All slides were submerged in solutions at 37°C unless otherwise stated. The volume varied depending on the number of slides run per experiment.

Day 1

Once chosen, sample sections were deparaffinized using 2, 15 minute xylene washes. Samples were then rehydrated with 2 minute washes in EtOH (100%, 95%, 70% and 50%), H₂O, and phosphate buffered saline (PBS). The tissue was fixed post PBS with 4% paraformaldehyde for 20 minutes. Tris buffered saline (TBS) normalized tissue pH and washed residual paraformaldehyde in a 5 and 10 minute wash. The sections were then subjected to a 10 minute 200mM HCL wash, followed by two TBS washes lasting 5 and 10 minutes. A 10 minute Acetic

Anhydride wash (25ml Tris pH 8, 224ml H₂O, 1.25ml Acetic Anhydride) created a uniform negative charge on the slide surfaces. After two TBS washes (5, 10 minute), the samples were subjected to a 15 minute, 10ug/ml Proteinase K wash in Tris-EDTA buffer, permeabilizing the tissues. Three 4°C TBS washes (5,10,20 minute) followed. Tissues were then subjected to probe incubation.

Using information gathered from the T7 sequencing reactions, control (sense orientation) and experimental (antisense orientation) probes were determined. Using the earlier dot blot, all probes were normalized to the activity expressed by 100 ng/ml of DIG control probe, and diluted 1:50 with hybridization buffer. In addition to the initial 1:50 dilution, Matriline's concentration was increased to 1:30 for optimization. Hybridization buffer was prepared as 2x SSC, 50% formamide, 10% dextran sulfate, 0.02% SDS, and 0.01% yeast tRNA. All hybridization reactions utilized 1.5 µl of probe in 75 µl of hybridization buffer except for matriline-4, which utilized 2.5 µl of probe. Once added to tissue sections, samples were coverslipped and heated to 95°C for 4 minutes. Slide were then sealed with rubber cement prior to over night (ON) hybridization at 55°C in the hybridization oven (Boekel). All slides were hybridized at 55°C. In addition to the 55°C initial temperature, matriline-4 was hybridized at 42°C.

Day 2

Rubber cement was carefully removed using forceps, and cover slips were passively lifted by submersing the samples in 2X SCC prewarmed to 55°C. Once the coverslip lifted, slides were placed into 55°C, 1:1, 2X SCC and formamide. Three 20 minute washes occurred in the 2X SSC/formamide solution at 55°C with gentle shaking. Two 15 minute SSC washes followed at RT shaking at 60 RPM (Labnet). Afterward, 3 TBS washes (5,10, 10 minute) at RT

normalized pH and removed residual probe from the tissues, preparing them for the Blocking agent and anti digoxigenin labeling. Sections were outlined using a pap pen, minimizing the volume of blocking agent and anti-DIG immunoreagent needed. The samples were incubated for 1 hour at RT in a Roche blocking agent (100mM maleic acid, 1x block stock, 150mM NaCl pH 7.5) containing 10% (fetal calf serum) FCS and 1% sheep serum (SS). Afterward, the sections incubated another hour in 1:1250 sheep anti-digoxigenin alkaline phosphatase (Roche) diluted with blocking buffer (Roche) containing 10% (FCS). Following 4 TBS washes (5,10,10,10 minute), sections with labeled probe were developed using NBT/BCIP substrate (Roche). While development time varied depending upon the probe, most sections were allowed to develop covered for 5-6 hours at RT before being transferred to 4°C ON.

Day 3

The following morning, the NBT/BCIP reaction was stopped by immersing the samples in ddH₂O. The sections were rinsed 3 times (5,10,10 minute), and 10 minute washes in 50% EtOH, 70% EtOH, and PBS followed. Following post fixation, sections were hydrated in 80% glycerol and 20% PBS and cover-slipped. Slides were then sealed using clear nail polish and slides were stored horizontally in a dark, RT environment. Images were taken using an Olympus BX41 microscope and DP72 camera, exposure remained consistent between experimental and control slides. Data regarding transcript expression were deemed sufficient if consistent localization and intensity was observed in two different embryos, with a minimum of 7 replicate sections per gene.

3. RESULTS

In situ hybridization was performed on developing HH35 (e9) chick limbs to investigate spatial expression patterns of extracellular matrix genes identified in the 2010 microarray study. Primers for each gene of interest were developed with the use of the NCBI database (Table 2), and specificity was established by BLAST analysis of the primer sequences against the *Gallus gallus* genome. Amplicons were inserted into a plasmid vector, purified (Fig 3), and sequenced (Fig 4) to confirm specificity and identify orientation of the insert. This allowed us to determine the proper restriction enzyme and polymerase needed to synthesize the control (sense) and experimental (antisense) probes. In all cases, Nde1 digest and SP6 polymerase synthesized control probes, and Nco1 digest and T7 polymerase synthesized experimental probes. Each probe's labeling activity was tested using a dot blot with all probe concentrations standardized to match the labeling intensity produced by 100 ng/ml of digoxigenin (DIG) labeled control RNA (Roche).

Images of serial control and experimental sections were photographed collectively under the same exposure with picture size, resolution, and contrast remaining consistent for all images.

Thrombospondin-3 (THBS3) Expression

Thrombospondin-3 (THBS3) mRNA localization showed significant expression when compared to its control (Fig 5). In the developing elbow, transcript localization was found along the joint interzone and expression was strongest within the presumptive articular cartilages surrounding the proximal humerus and distal ulna. In the ulna THBS3 localization was present along the perichondrium of the diaphysis, with little expression extending into underlying chondrocytes. Superficial to the developing skeleton, dermal and skeletal muscle tissue also illustrated moderate transcript levels.

With increased magnification (Fig 6), intensity differences became apparent in the elbow and ulna. The highest degree of expression in the elbow was concentrated to the developing articular cartilages, with levels declining toward the interzone and epiphyseal ends of the ulna and humerus (Fig 6 C,E,G). Higher magnification of the ulna (Fig 6 D,F,H) depicted localization particularly in the inner layer of the perichondrium, closest to the chondrocytes. Further magnification in both instances demonstrated specific intracellular transcript expression.

Discoidin Domain Receptor-2 (DDR2) Expression

Discoidin Domain Receptor-2 (DDR2) illustrated high expression throughout the developing chick limb when compared to its control (Fig 7). Transcript localization in the developing elbow was concentrated along the presumptive articular surfaces with weaker signal comprising the central interzone. The expression pattern in the carpal joint however was considerably different with robust mRNA expression extending throughout the interzone. DDR2 transcripts were also depicted in moderate levels throughout the perichondrium in the ulna and within chondrocytes of the skeletal primordium. Lateral to the ulna, DDR2 mRNA was also evident in skeletal muscle masses surrounding the developing joints, and extending throughout the limb. In the dermis intense asymmetric localization was identified on the posterior side of the developing limb with a substantial decrease in the underlying connective tissue.

With increased magnification (Fig 8.), expression differences between more proximal and distal tissues, and intracellular localization of mRNA transcripts became evident. DDR2 transcripts within the elbow were conserved primarily to the presumptive articular regions of the distal humerus, proximal ulna, and proximal radius (Fig 8 D,G,J). A distinct decline of expression intensity was observed within the central lamina of the interzone. This is contrary to the robust signal encompassed within the interzone and articular region of the carpal joint (Fig 8

E,H,K). A decrease in expression was only detected moving into the subjacent chondrocytes in the head of the ulna. Comparing expression within the elbow and carpal joints, a distinct proximal-distal gradient in mRNA localization is evident with greater levels found within the carpal joint. DDR2 transcripts were also highly localized in the posterior ectoderm and dermis, with moderate expression in developing skeletal muscle masses (Fig 8 C,F,I).

In situ hybridization performed on earlier stage wings HH34 (e8) illustrated similar transcript localization when compared to the control (Appendix A). The interzone and presumptive articular cartilage portrayed similar expression levels compared to lower expression in the epiphyseal ends of the humerus and ulna (Appendix B). Similar to E9, the dermis and perichondrium showed a high degree of expression. mRNA also extended into underlying chondrocytes and surrounding skeletal muscle at moderate levels.

DDR2 expression in embryo sections at HH25 (e5) illustrated localization in several tissue types compared to the control (Appendix C). Moderate detection was observed in the presumptive dermis of the developing hind limb, notochord, and mesonephros of the developing kidney, with stronger expression evident in the sensory retina and developing brain vesicles (Appendix D).

Sushi Domain Containing 5 (SUSD5) Expression

Sushi Domain Containing 5 transcripts illustrated broad expression throughout the chick limb when compared to its control (Fig 9). Elbow joint mRNA localization was present along the presumptive articular surfaces of the humerus, radius, and ulna but did not extend appreciably into the interzone. Moving distally to the carpal joint, transcript levels appeared to be uniform throughout the interzone and developing articular region. Robust SUSD5 expression was

substantial in the perichondrium of the radius and ulna extending into underlying chondrocytes. In lateral soft tissues, localization was only apparent in skeletal muscle.

Increased magnification (Fig 10) enhanced SUSD5 transcriptional expression differences in regions of interest. In the elbow, asymmetrical staining was apparent within the articular region, with a higher degree of localization found at the developing olecranon process (Fig 10 D,G,J). In the carpal joint, asymmetric expression was illustrated along the medial edge of the distal radius (Fig 10 E,H,K). Comparing the interzone transcript level in the elbow to that of the carpal joint, a clear proximal-distal expression gradient was observed with a higher degree of localization found at the carpal interzone. This gradient was reversed however when evaluating expression within developing articular cartilage, with the elbow showing greater mRNA localization. In the radial shaft magnification illustrated higher transcript levels with lower levels in subjacent chondrocytes (Fig 10 C,F). Skeletal muscle cells also showed moderate SUSD5 expression throughout the developing limb (Fig. 10 I). Higher magnifications also depicted intracellular detection demonstrating specific mRNA localization.

In situ hybridization performed on (e5) embryo sections localized SUSD5 mRNA at restricted sites in the trunk, specific to the peripheral cells of the notochord (Appendix E).

Matrix Remodeling-Associated 5 (MXRA5) Expression

Matrix Remodeling-Associated 5 (MXRA5) mRNAs illustrated strong, but restricted expression domains in the e9 wing joints when compared to its control (Fig 11). mRNA transcripts in the forming elbow region were detected at moderate levels in the interzone with higher levels comprising the presumptive articular cartilages. Surrounding the developing joint, moderate expression was also seen in skeletal muscle. MXRA5 expression in the carpal joint was confined to the interzone and developing articular surface with similar signal intensity.

Increased magnification of both the elbow and carpal regions (Fig12) illustrated asymmetric localization of MXRA5 transcripts. Within the elbow, mRNA was abundant at the presumptive articular surface of the developing coronoid process (Fig 12 C,E,G). This expression decreased in the central laminae of the interzone. Examination of the carpal joint depicted asymmetry of mRNA expression showing strong signal toward the medial articular surface of the radius (Fig 12 B,D,F,H). Higher magnifications showed intracellular cytoplasmic staining confirming intracellular localization of transcript expression.

Matrilin-4 (MATN4) Expression

Matrilin-4 (MATN4) showed little expression when compared to its control (Fig 13). Detection of transcripts in the elbow was minimal with only low-level localization in the articular region. The ulna illustrated slight expression along the perichondrium, with slightly increased expression localized in the dermis.

Higher magnification also displayed the weak to moderate MATN4 expression within the limb (Fig 14). The elbow depicted faint expression along the articular cartilages in the elbow joint (Fig 14 D,G,J). As magnification increased, the purple coloration signifying MATN4 transcripts was hardly visible. Localization in the perichondrium was detected with mild expression, but was not present in neighboring chondrocytes (Fig 14 E,H,K). Detection in the dermis showed a slightly increased level of MATN4 expression, highest in the covering ectoderm (Fig 14 C,F,I).

Summary

In situ hybridization of each of the five genes depicted similarities and differences in the degree of expression and area of localization within the e9 chick wing tissues (Table 5). In the developing elbow joint, THBS3, SUSD5, and MXRA5 all showed robust expression levels,

while DDR2 and MATN4 showed moderate and faint expression, respectively. In the carpal joint, DDR2, SUSD5, and MXRA5 depicted robust mRNA expression, however THBS3 and MATN4 were not evaluated. Detection within muscle masses showed moderate transcript levels of DDR2, SUSD5, and MXRA5, with only faint expression of THBS3 and MATN4. Within the perichondrium, SUSD5 displayed strong mRNA levels, while THBS3 and DDR2 presented slightly weaker expression. MATN4 exhibited faint expression and MXRA5 depicted little to no localization in the perichondrium. Lastly in underlying chondrocytes, SUSD5 illustrated moderate expression levels, with THBS3 and DDR2 only showing faint localization. Both MXRA5 and MATN4 showed little or no specific signal.

Table 2. Genes, accession numbers, primer sequences and probe lengths for *in situ* hybridization.

Gene	Accession #	F. Primer	R. Primer	Sequence Length
THRB3	L81165.1	GAAATGAGCAACCC CACCCA	GTCGCTGTTCATGGT CTGCA	460
DDR2	XM_422211.3	ACTCCAACCCCTACG ACATC	TCGTTGGCATCGGAT CGGAA	505
SUSD5	XM_418822.3	GACTTCGTGGCGGGG CGATG	ACGCCGTCTCCTTGT TGCCC	537
MXRA5	XM_416853.3	AATTTACTCCAGCTT CATCC	CATTGCCATGCTCAT CAGTC	490
MATN4	XM_425698.3	AAGTCCTGTCGCGCC ATT	TTGGGAGCGTCCATC CGTGAAG	510

Table 2. The accession number for each gene led to the cDNA sequence. This was used to produce the forward and reverse primers, which encompassed the transcript sequence used for probe generation.

Figure 3. Gel electrophoresis data confirms approximate insert size.

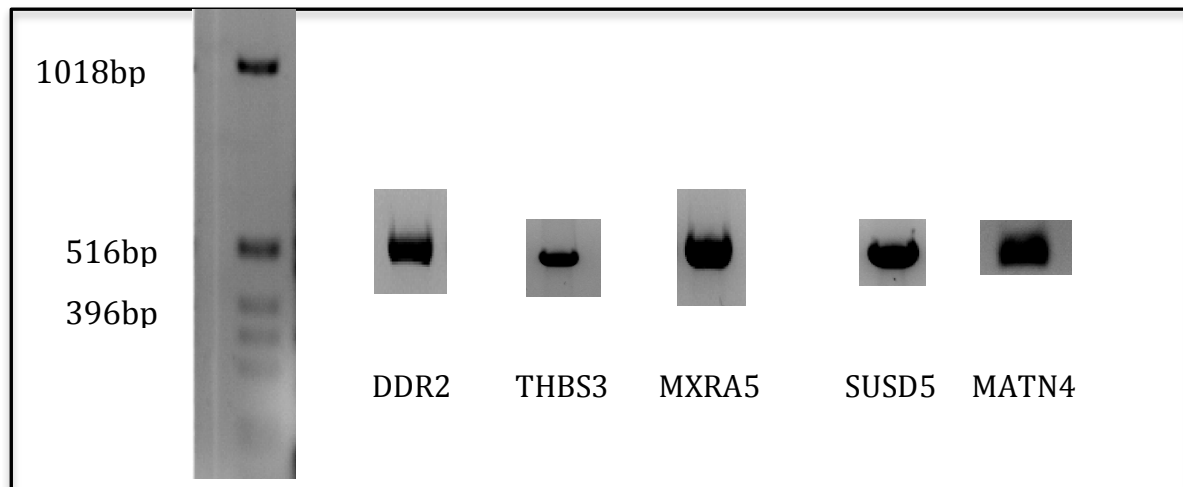


Figure 3. Gel electrophoresis data illustrates insert size. Bands confirm that inserts match the initial transcript size determined during primer development. The corresponding gene is noted below the insert and all transcripts are referenced to a 1kb ladder.

Figure 4. Confirmation of cloned insert sequences based on DNA sequencing
using T7 sequencing primers

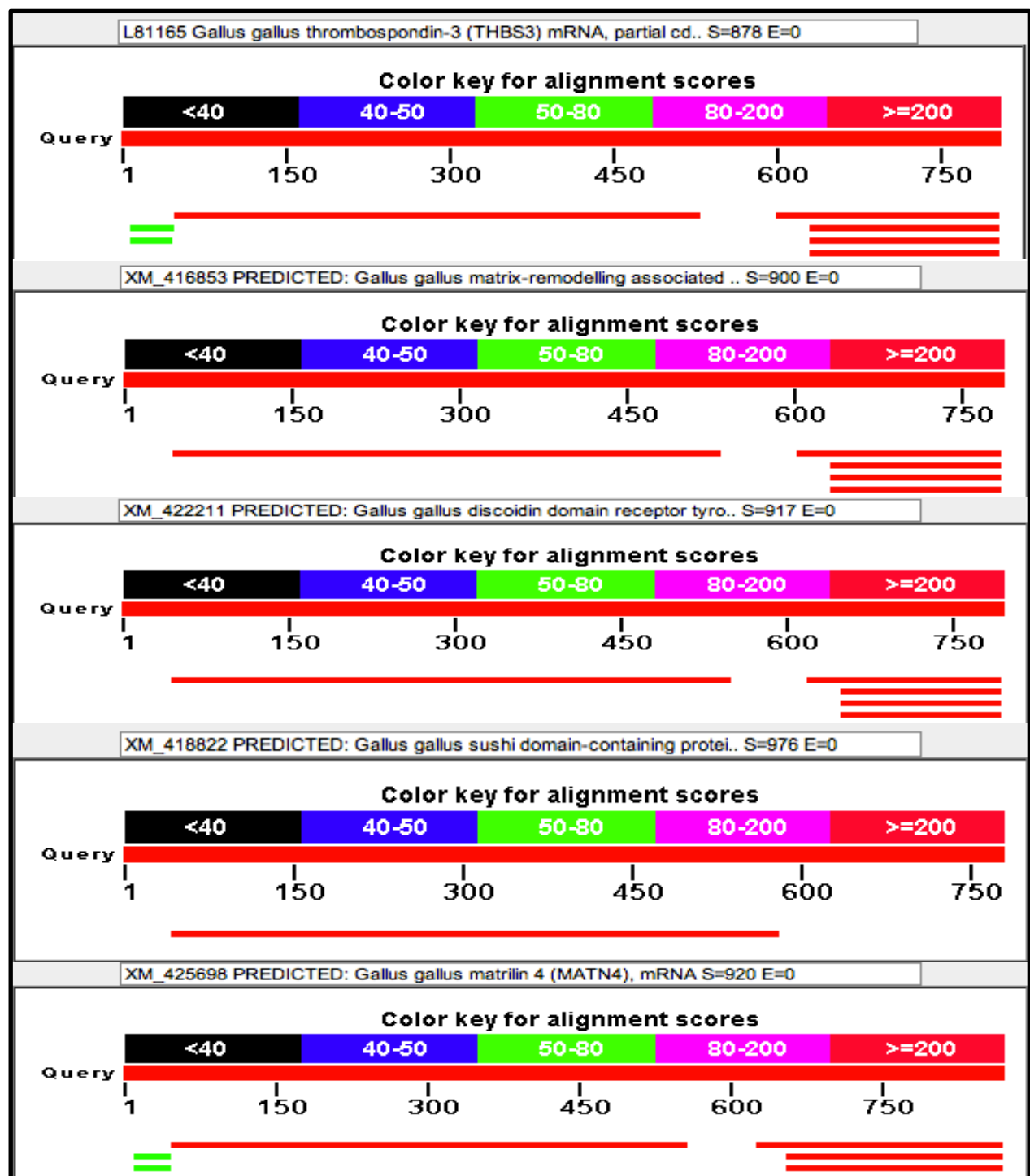


Figure 4. The sequence identity of each plasmid insert corresponds to the respective gene. Alignment scores illustrate the transcript location within the mRNA sequence and single red line depicted represents correct sequence length and specificity limited to only the corresponding gene.

Table 3. Nano-drop quantitation of purified plasmids

Gene	Concentration (ng/μl)	260/280
THRB3	176.5	1.86
DDR2	154.4	1.84
SUSD5	290	1.94
MXRA5	144	1.94
MATN4	178.6	1.96

Table 3. Nano-drop results give concentration levels sufficient for probe generation. The concentration is referenced in ng/μl, and the 260/280 is a measure of sample purity.

Figure 5. Thrombospondin-3 (THBS3) *in situ* hybridization in serial sagittal sections of chick wing at HH35 (e9).

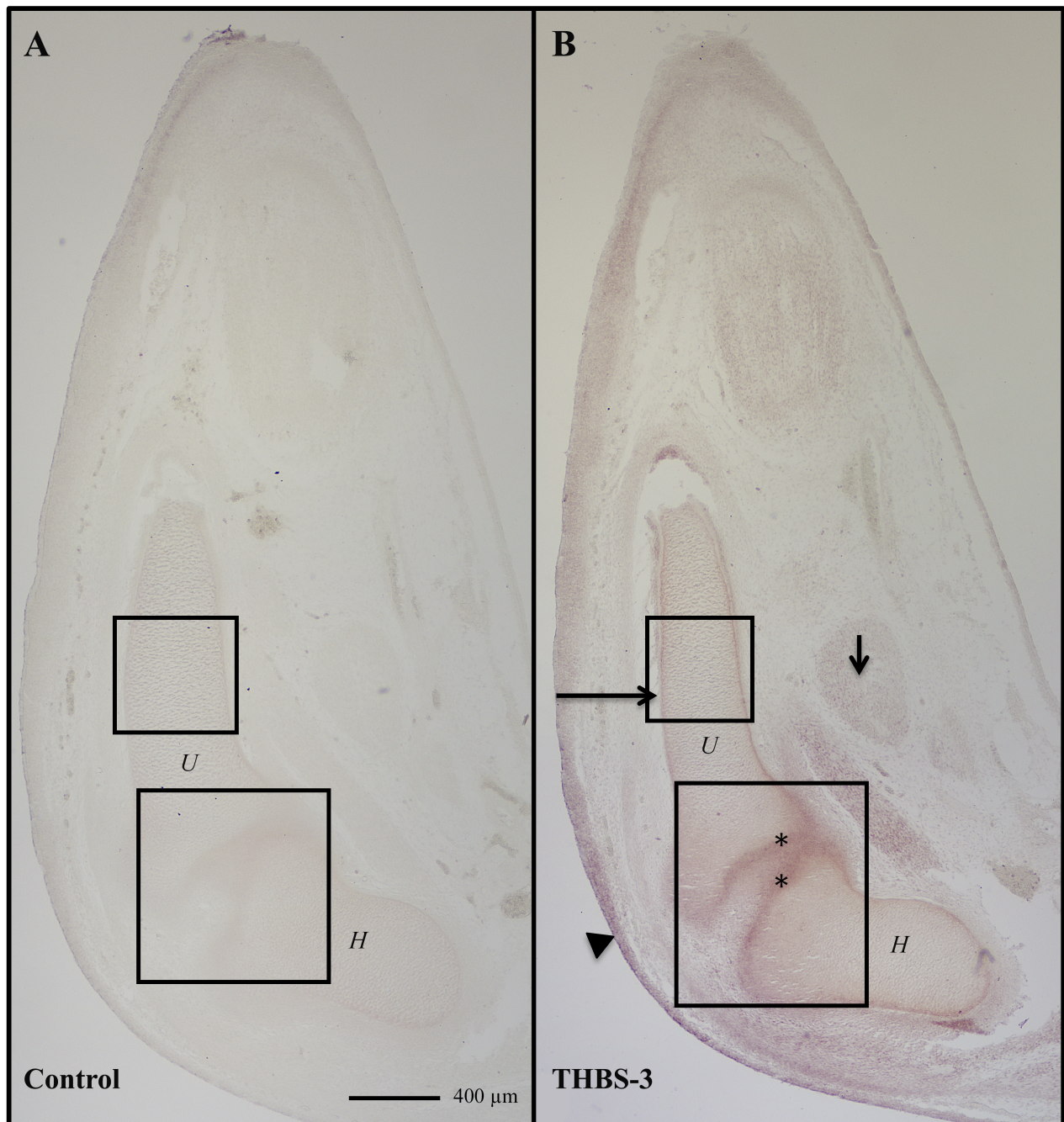


Figure 5. Thrombospondin-3 (THBS3) *in situ* hybridization in serial sagittal sections of chick wing at HH35 (e9). A: Little or no signal detection present in control limb tissues hybridized with the sense probe. B: THBS3 mRNA localization is robust at future articular surfaces (*) of the humerus (*H*) and ulna (*U*), with moderate transcript expression observed in the perichondrium (long arrow). Mild to moderate mRNA detection present in the interzone, skeletal muscle (short arrow), ectoderm (arrowhead), and underlying dermis. Regions containing substantial expression (boxed) are magnified in Fig 6. Scale bar in A= 400 μ m for A & B.

Figure 6. THBS3 areas of interest under increasing magnification.

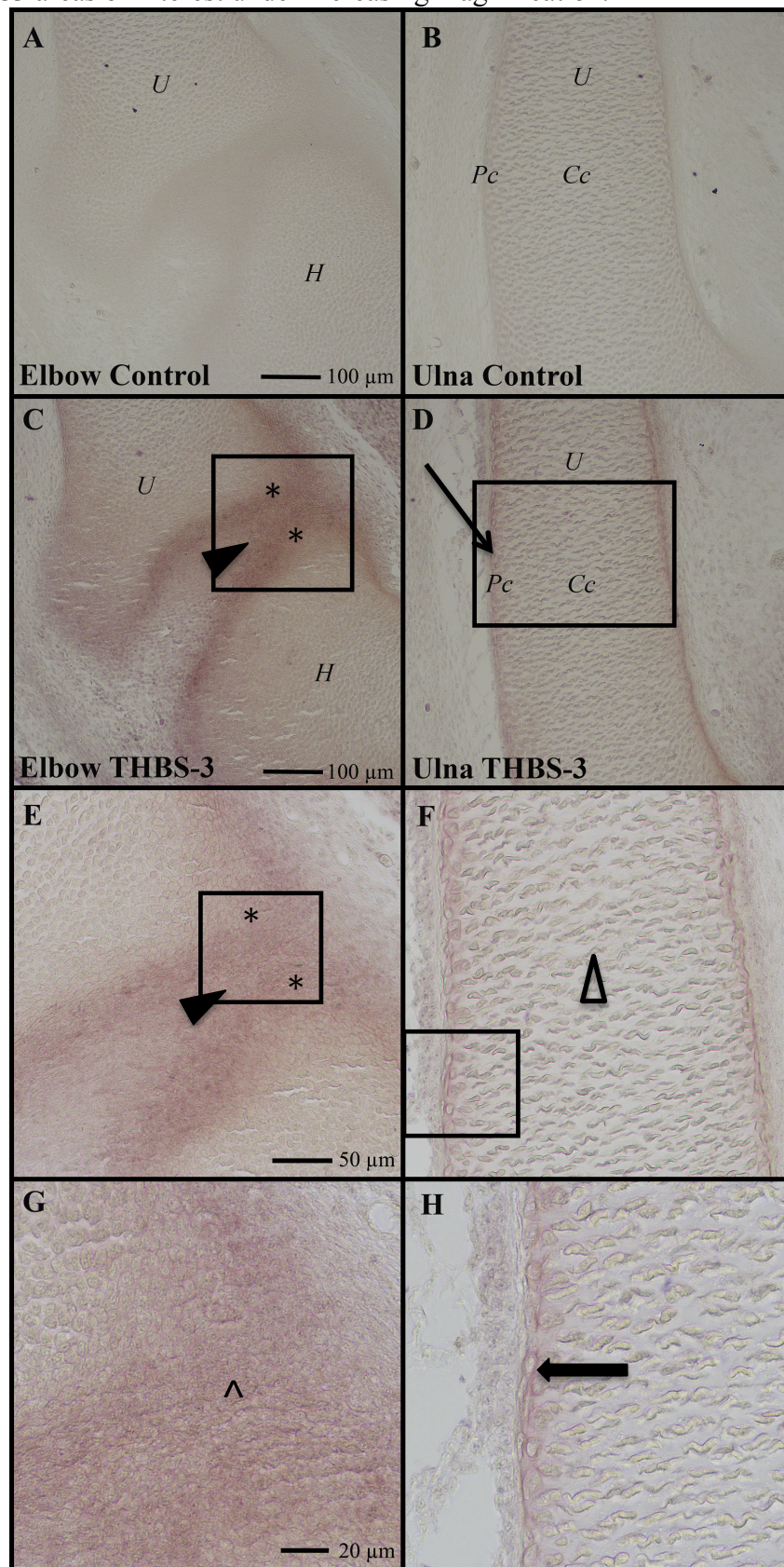


Figure 6. THBS3 areas of interest under increasing magnification. Elbow (left) and ulna (right) illustrate THBS3 transcript localization. Controls (A and B) show little or no specific staining. Strong transcript levels in the elbow (C) are present in humeral (*H*) and ulnar (*U*) articular cartilages (*) as well as the interzone (arrowhead). Higher magnification of the boxed region displays (E) showing distinct articular expression (*) with decreasing levels toward the interzone (Arrow) and epiphyseal ends of ulna and humerus. Further magnification (G) reveals cytoplasmic THBS3 mRNA (caret). Ulnar expression (D) is strongest in the perichondrium (*Pc*)(Arrow). Magnifying the boxed region, (F) displays THBS3 along the inner layer of the perichondrium in chondroblasts (boxed) extending into underlying chondrocytes (*Cc*)(empty arrowhead). (H) Denotes higher magnification of the region boxed in (F), revealing transcript intensity differences between the (*Pc*) and (*Cc*). Scale bar in A= 100 μ m for A-D. Scale bar in E= 50 μ m for E&F. Scale bar in G&H= 20 μ m.

Figure 7. Discoidin Domain Receptor-2 (DDR2) *in situ* hybridization in serial sagittal sections of chick wing at HH35 (e9).

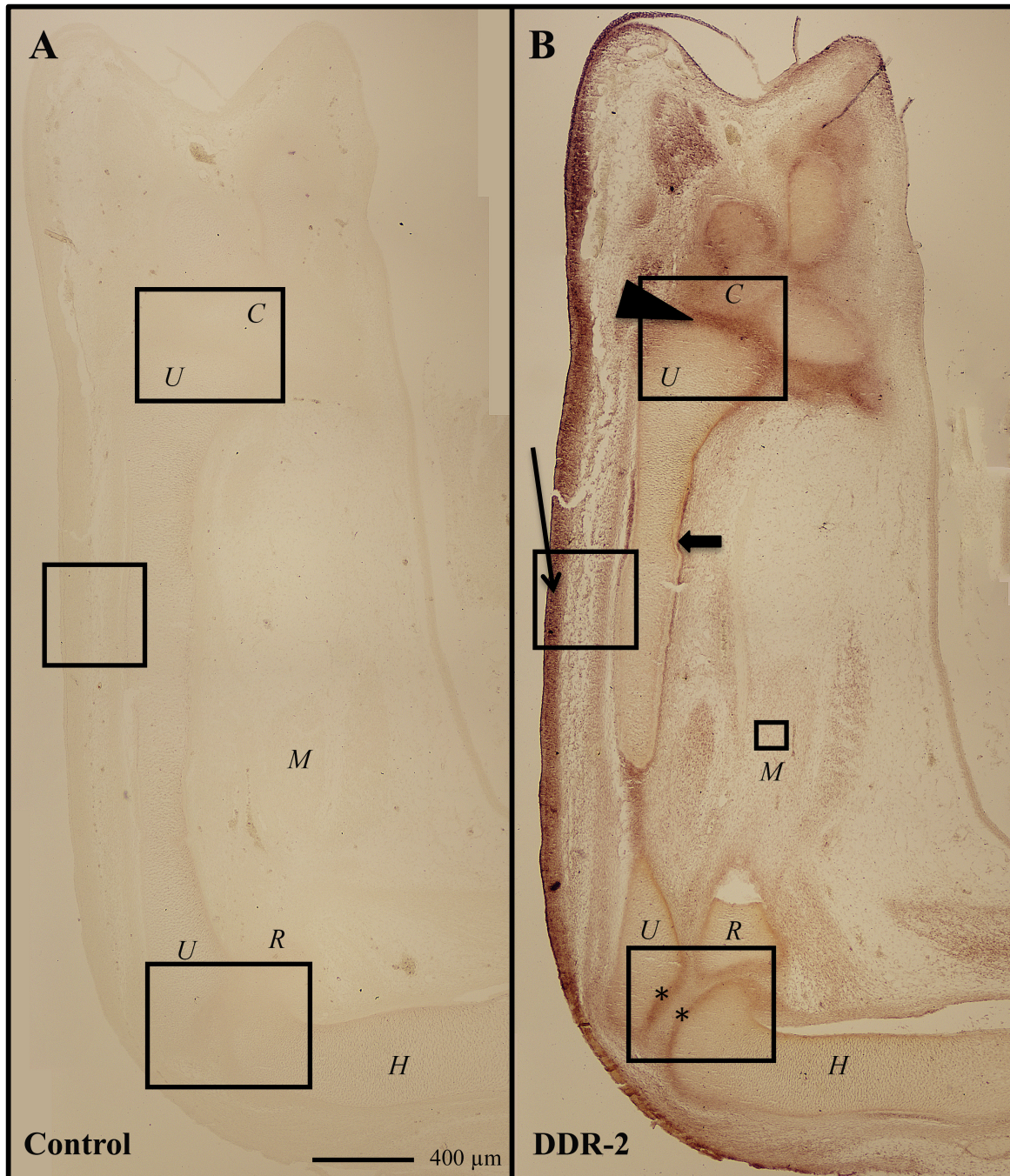


Figure 7. Discoidin Domain Receptor-2 (DDR2) *in situ* hybridization in serial sagittal sections of chick wing at HH35 (e9). A: Little or no transcript detected in the control limb tissues hybridized with the sense probe. B: In the elbow, moderate to high levels of DDR2 transcripts were present in presumptive articular cartilages (*) of the distal humerus (*H*), proximal radius (*R*), and proximal ulna (*U*). carpal joints (*C*) display high levels of transcript within the interzone (arrowhead). Expression extended into the ectoderm and dermis (arrow), along with skeletal muscle (small box) and developing ulna (bold arrow). Areas of strong mRNA localization (boxed) are magnified in Fig 8. Scale bar in A= 400 μ m for A & B.

Figure 8. DDR2 areas of interest under increasing magnification.

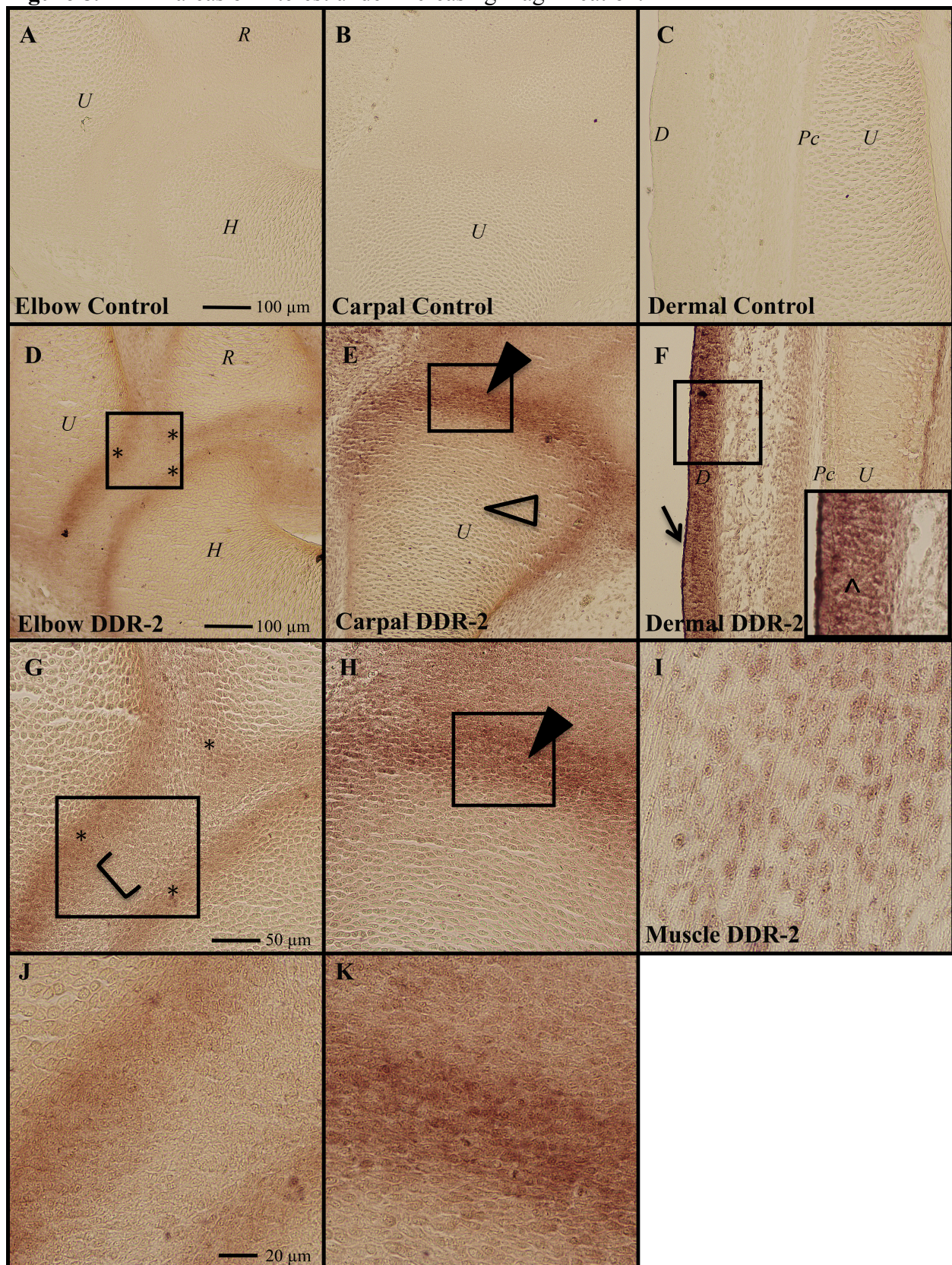


Figure 8. DDR2 areas of interest under increasing magnification. Controls (A-C) show little or no specific DDR2 detection with minimal background. Experimental groups: Elbow (left column), carpal (middle column), Dermal (C, F) and skeletal muscle (I) show moderate to significant DDR2 mRNA localization. Transcript expression in the elbow (D) is concentrated in the presumptive articular cartilage (*) of the humerus (*H*), ulna (*U*), and radius (*R*). (G) Depicts higher magnification of the area boxed in (D), showing less mRNA in the interzone (bracket). Increased magnification (J) of boxed region in (G) illustrates intracellular differences. DDR2 expression in carpal joint (E) is exhibited throughout the interzone (filled arrowhead), with moderate expression in chondrocytes (empty arrow). (H) Depicts higher magnification of the boxed area in (E), showing strong transcript localization (filled arrowhead). (K) is a further magnification of boxed area in H. Dermal (*D*) and ectoderm (arrow) transcript expression was robust (F), and increased magnification within the inset displayed high intracellular localization (caret). The perichondrium (*Pc*) showed moderate DDR2 expression along with skeletal muscle (I). Scale bar in A= 100 μ m for A-F. Scale bar in G= 50 μ m for G&H. Scale bar in I= 20 μ m for I-K, and small image in (F).

Figure 9. Sushi Domain Containing 5 (SUSD5) *in situ* hybridization in sagittal sections of chick wing at HH35 (e9).

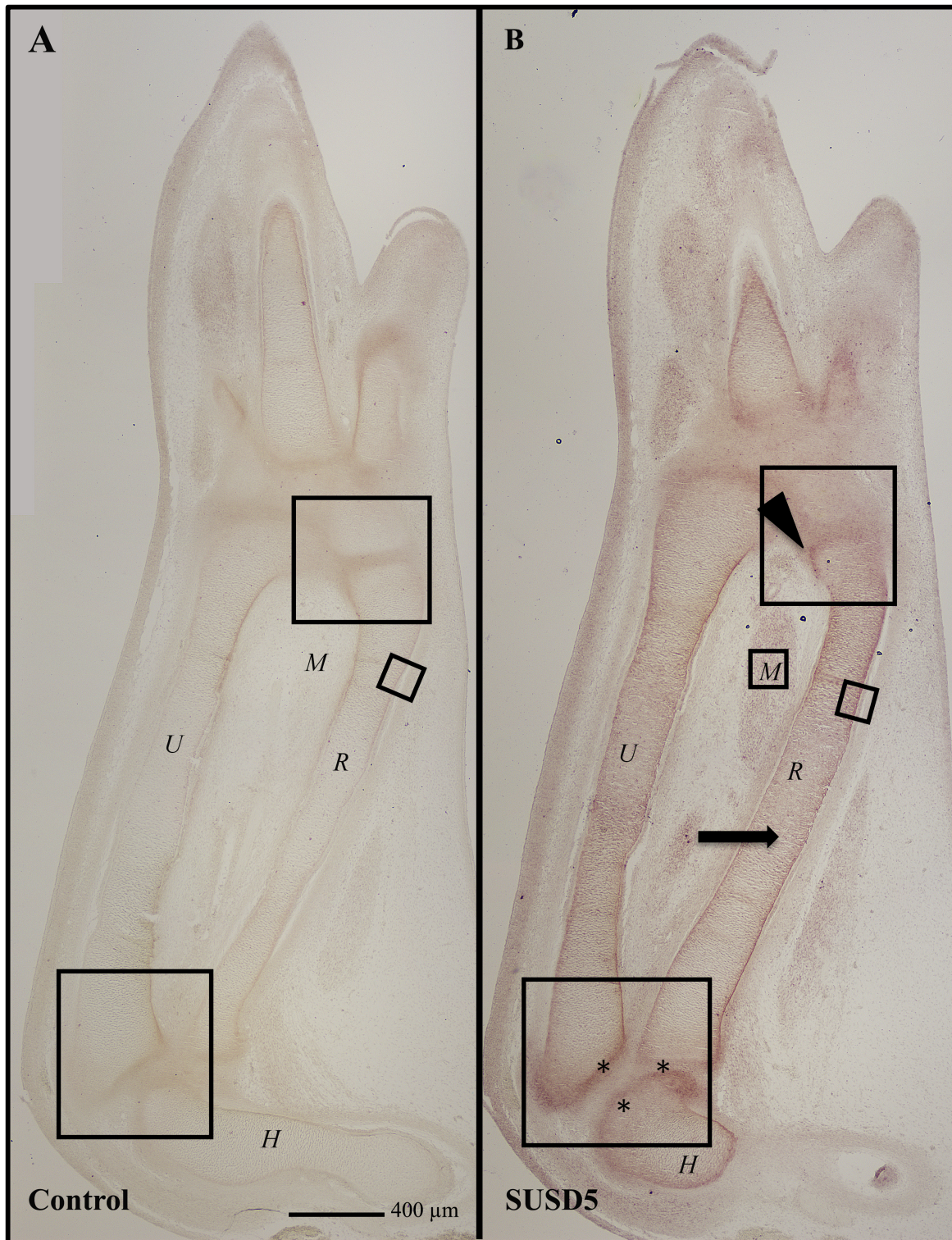


Figure 9. Sushi Domain Containing 5 (SUSD5) *in situ* hybridization in serial sagittal sections of chick wing at HH35 (e9). A: Little signal is present in control limb hybridized with the sense probe. B: Elbow illustrates robust SUSD5 expression in developing articular cartilages (*) of distal humerus (*H*), proximal ulna (*U*) and proximal radius (*R*). Localization extends into the perichondrium (arrow) and chondrocytes (bold arrow). Moderate SUSD5 expression comprises the carpal interzone with asymmetry at presumptive articular surface (arrowhead). Transcripts extend into skeletal muscle (*M*). Regions of substantial mRNA localization (boxed) are magnified in Fig 10. Scale bar in A= 400 μ m for A & B.

Figure 10. SUS D5 areas of interest under increased magnification.

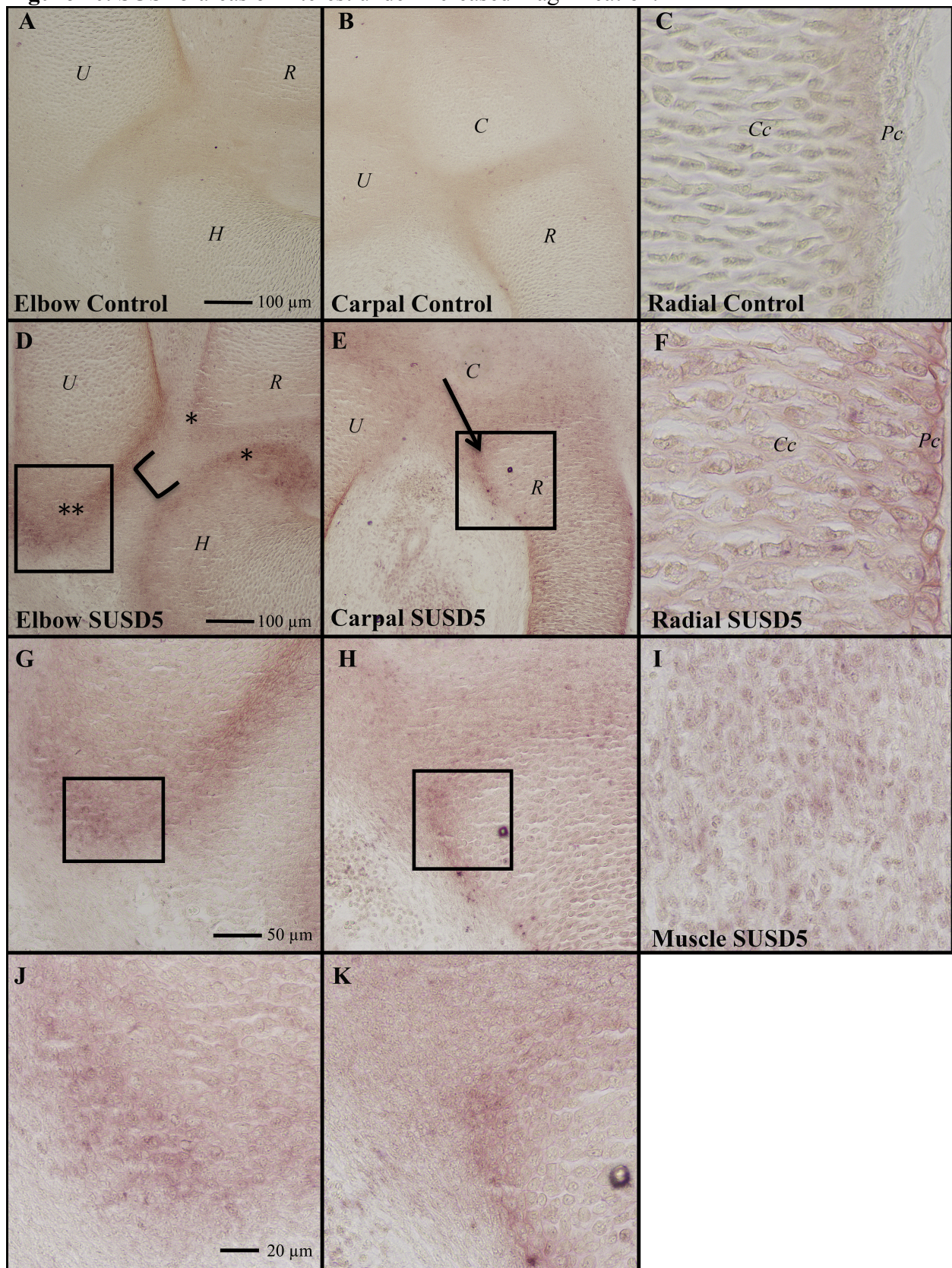


Figure 10. SUS D5 areas of interest under increased magnification. Controls (A-C) correspond with boxed areas of interest (Fig 9A) showing little to no specific signal and low background. Experimental sample images (D-K) organized by column: left- elbow(A,D,G,J) middle-carpal (B,E,H,K), right- radius (C,F) and muscle (I). SUS D5 transcripts in the elbow (D), show little signal in the interzone (bracket) with asymmetrical localization (**) in the articular region (*) of the distal humerus (*H*), proximal ulna (*U*) and proximal radius (*R*). The boxed region in (D) is magnified (G) demonstrating expression in olecranon process. (J) is a further magnification. The carpal joint (E) contains abundant expression. (H) Shows higher magnification of the area boxed in (E) demonstrating asymmetrical patterning on medial radius. (J) is a further magnification of the boxed region in (H). The radius shows mRNA localization (F) in the perichondrium (*Pc*), and within the chondrocytes (*Cc*). Differentiating skeletal muscle myoblasts (I) also show moderate SUS D5 transcript localization. Scale bar in A= 100 μ m for A, B, D, E. Scale bar in G= 50 μ m for G&H. Scale bar in I= 20 μ m for C, F, I, J, K.

Figure 11. Matrix Remodeling-Associated 5 (MXRA5) *in situ* hybridization in serial sagittal sections of chick wing at HH35 (e9).

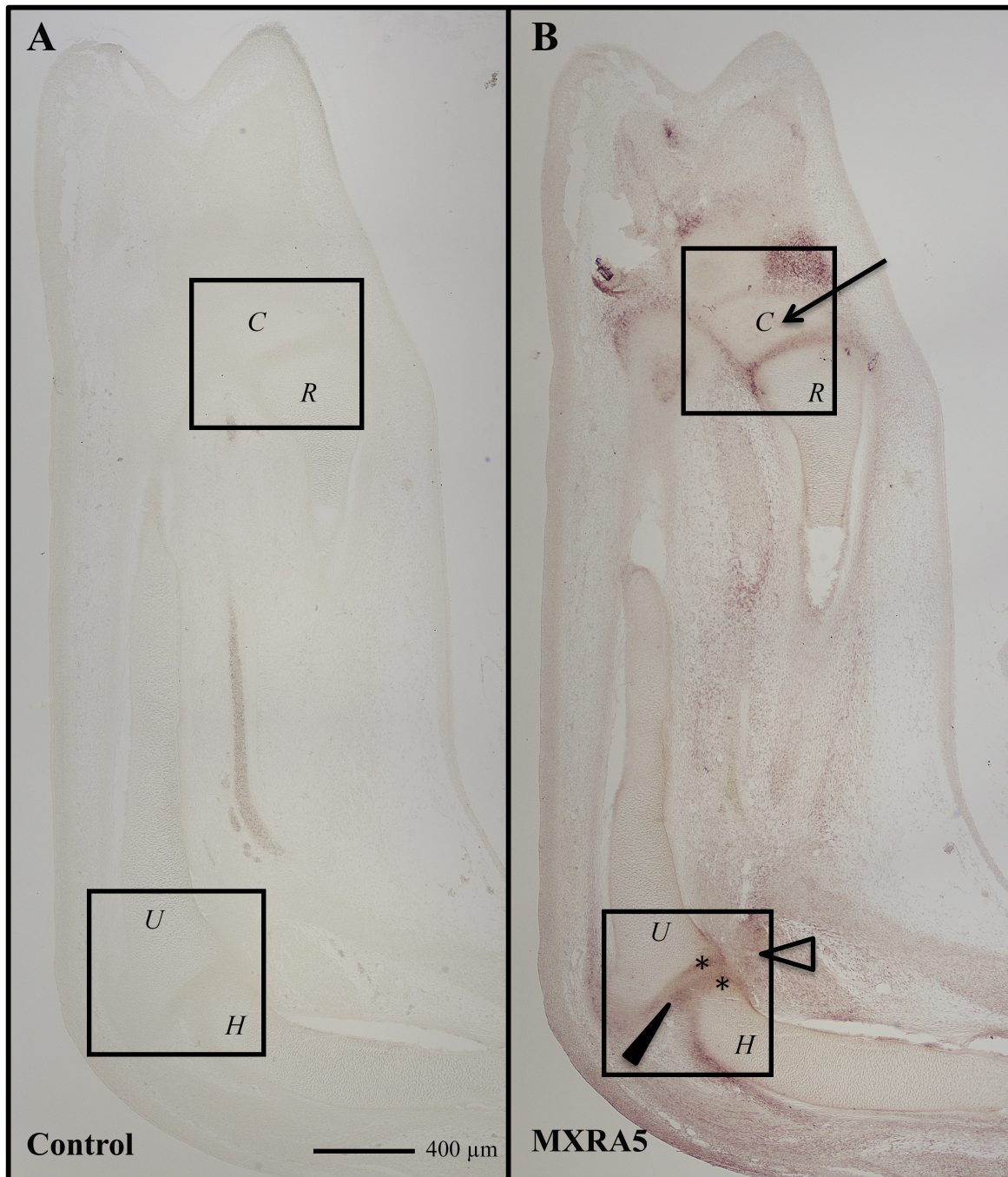


Figure 11. Matrix Remodeling-Associated 5 (MXRA5) *in situ* hybridization in serial sagittal sections of chick wing at HH35 (e9). A: Little to no transcript expression present in control limb hybridized with sense probe. B: Elbow displays asymmetric MXRA5 expression within presumptive articular surfaces (*) of proximal humerus (*H*), and distal ulna (*U*). Moderate expression found within the Interzone (filled arrowhead) and skeletal muscle (empty arrowhead). Transcripts intermediate to carpal (*C*) and radius (*R*) are localized to the interzone with visible asymmetry (arrow). Regions of interest (boxed) are magnified in Fig 12. Scale bar in A= 400 μ m for A & B.

Figure 12. MXRA5 areas of interest under increased magnification.

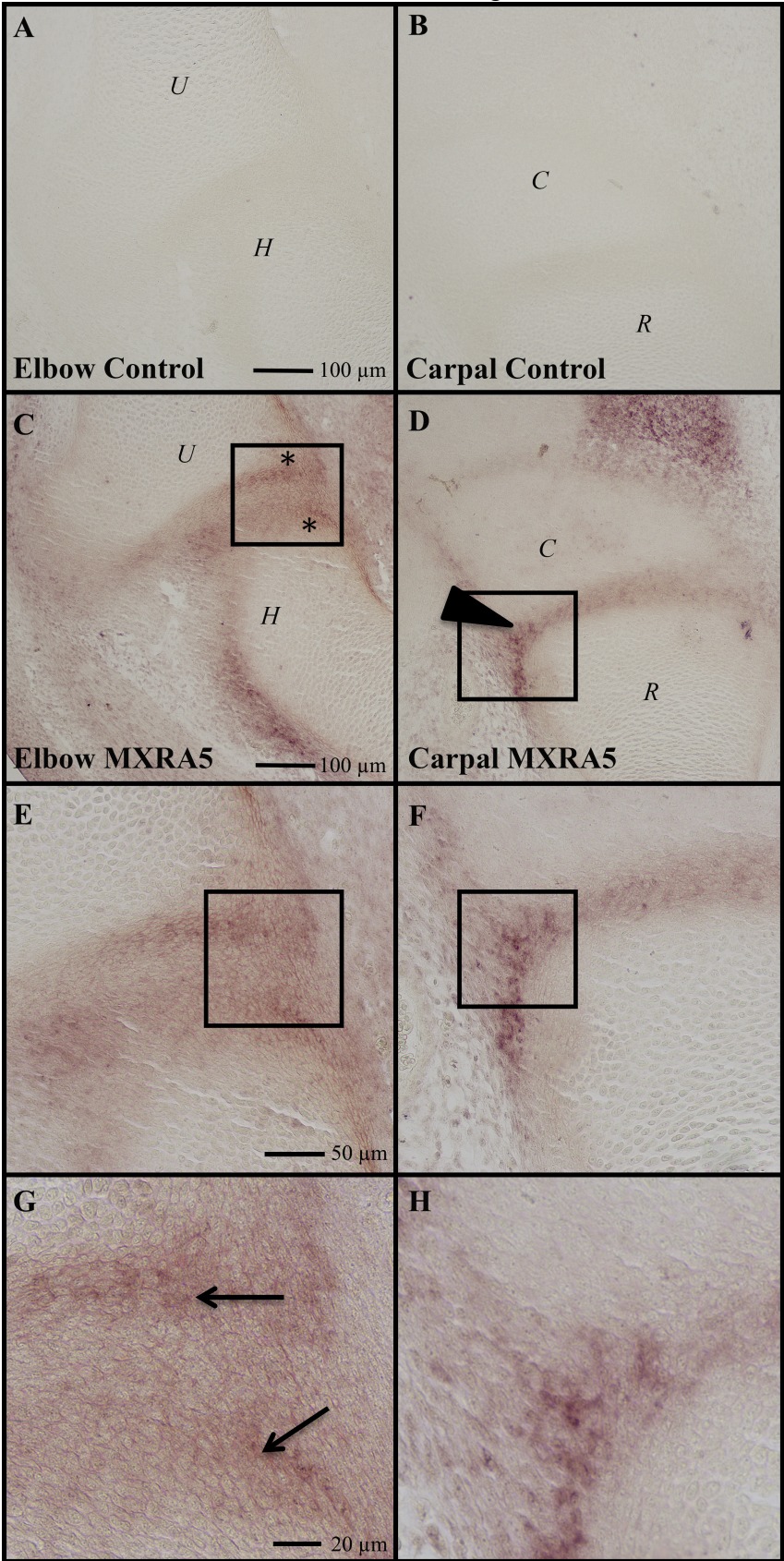


Figure 12. MXRA5 areas of interest under increased magnification. Controls (A-B) correspond to boxed regions from (Fig 11A) and show little or no transcript localization. Magnifications of experimental samples (C-H) are organized by column (left- elbow and right- carpal). Elbow localization (C) of MXRA5 expression is observed in the interzone with asymmetric expression in presumptive articular region (*) of the coronoid process. Increasing the magnification of the boxed area in (C), (E) illustrates a higher transcript level in the articular region as opposed to the interzone (bracket). Further magnification (G) demonstrates intracellular expression differences in articular tissue and interzone. Carpal joint (D) mRNA expression is noted in the interzone and articular cartilage with asymmetrical localization at the distal end of the radius (arrowhead). (F) Higher magnification of region boxed in (D) illustrates interzone expression. Boxed areas in (E) and (F) represent increased magnifications of interzone regions depicted in (G) and (H) respectively. Scale bar in A= 100 μ m for A-D. Scale bar in E= 50 μ m for E&F. Scale bar in I= 20 μ m for G&H.

Figure 13. Matrilin-4 (MATN4) *in situ* hybridization in serial sagittal sections of chick wing at HH35 (e9).

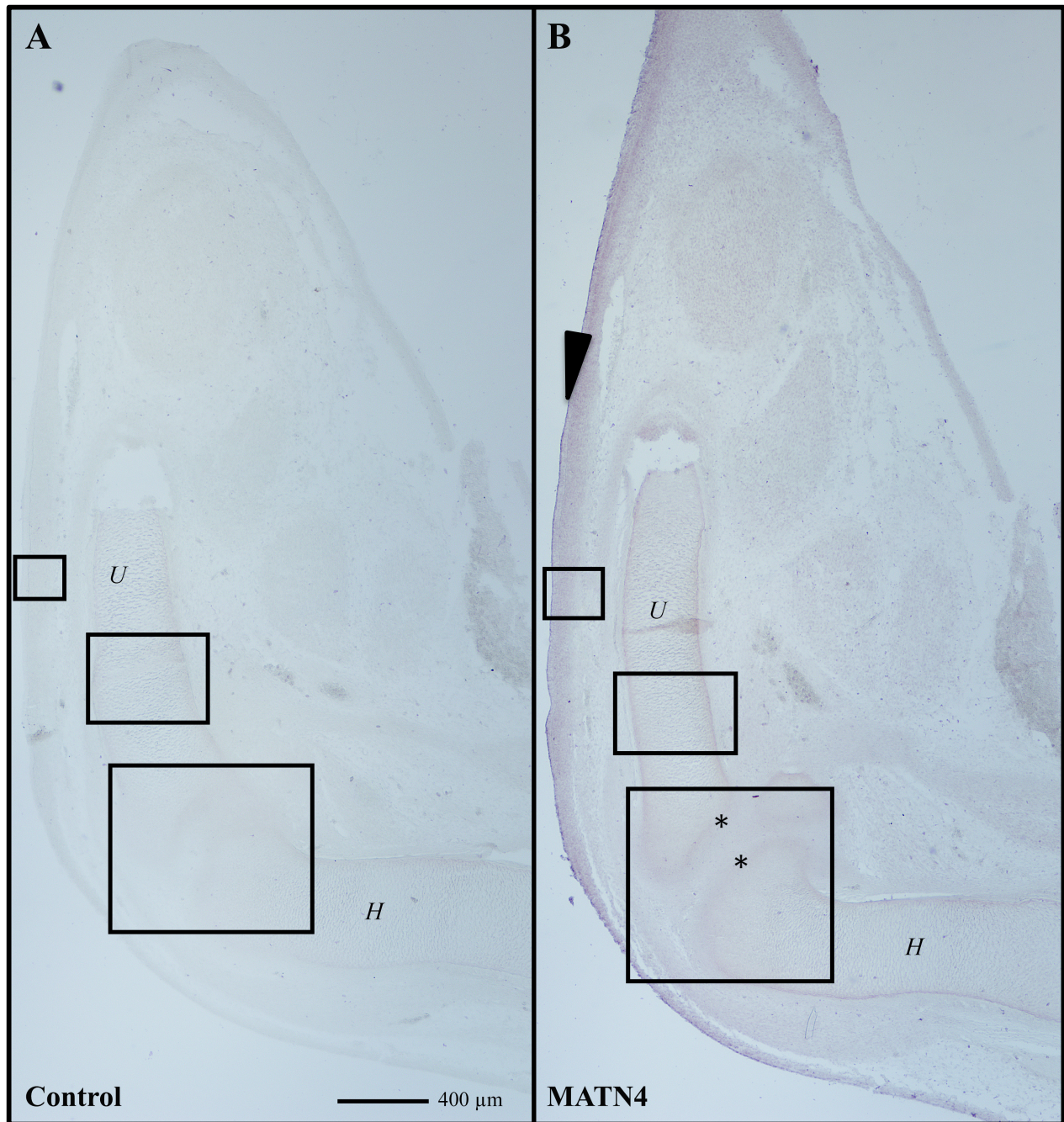


Figure 13. Matrilin-4 (MATN4) *in situ* hybridization in serial sagittal sections of chick wing at HH35 (e9). A: Little to no transcript expression present in the control limb. B: Only slight expression of MATN4 transcripts are visible in articular elbow joint cartilages (*) of the distal humerus (*H*), and proximal ulna (*U*). Weak MATN4 expression observed in perichondrium (arrowhead). Moderate transcript levels present in ectoderm and subjacent dermis. Areas of interest (boxed) are further magnified in Fig 14. Scale bar in A= 400 μ m for A & B.

Figure 14: MATN4 areas of interest under increased magnification.

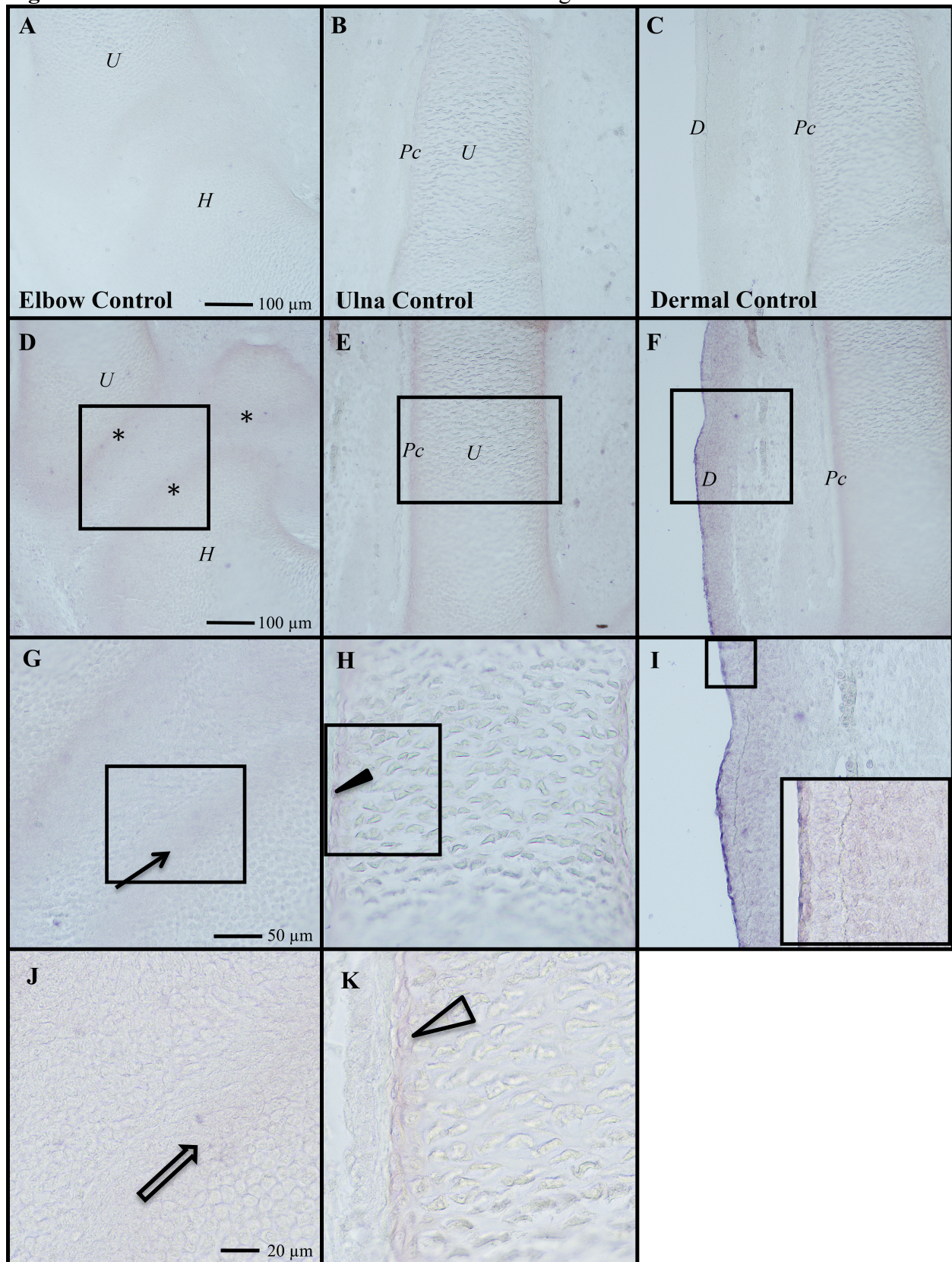


Figure 14. MATN4 areas of interest under increased magnification. Controls (A-C) correspond to boxed areas of in Fig 13A depicting little to no transcript detection. Experimental magnification (D-K) are organized by column (left- elbow, middle- ulna, right- dermis). Elbow joint expression shows little or no observable transcripts in the interzone (D) with weak localization in articular cartilages (*) of the distal humerus (*H*) and proximal ulna (*U*). When the boxed region is magnified (G), and further in (J), still only low level expression of intracellular transcripts are detected. Ulnar MATN4 expression is noted in the perichondrium (*Pc*). Further magnified, (H) depicts light staining limited to the inner lining of perichondrial cells (filled arrowhead). (K) Illustrates increased magnification of box region in (H) showing intracellular transcripts (empty arrowhead). Dermal (D) and ectodermal expression shows moderate level of MATN4 transcripts (F). Further magnification (I), demonstrates the degree of staining. When dermal localization is magnified (inset box) intracellular transcripts are evident. Scale bar in A= 100 μ m for A-F. Scale bar in G= 50 μ m for G-I. Scale bar in J= 20 μ m for J, K and inset box in I.

Table 4: Summary of mRNA expression for each gene categorized by tissue.

Genes	Elbow Joint	Carpal Joint	Dermis/ Ectoderm	Muscle	Perichondrium	Chondrocytes
THBS-3	+++	n/e	+	+	++	+
DDR-2	++	+++	+++	++	++	+
SUSD5	+++	+++	-	++	+++	++
MXRA5	+++	+++	-	++	-	-
MATN4	+	n/e	+	+	+	-

Table 4: Summary of mRNA expression for each gene categorized by tissue. The subjective chart displays expression levels in terms of no expression (-), faint expression (+), moderate expression (++), and strong expression (+++) and not examined (n/e)

4. DISCUSSION

The complex pathways and molecular interactions involved in creating a functional synovial joint are slowly being unraveled. Several key morphological steps are observed during this process. The formation of the interzone is a critical stage, marking the presumptive area where articular cartilages arise and physical separation of tissues (cavitation) will occur. Understanding the molecular mechanics involved in this transformation is critical to understanding joint development.

The intent of this study was to identify spatial expression patterns of extracellular matrix genes within the developing chick elbow joint at HH35 (e9). E9 marks the stage of interzone development at which cavitation in the elbow will soon begin. Genes of interest in the present study were first identified from a 2010 microarray experiment manipulating versican expression in the elbow during this same developmental period. Previous studies (Choocheep et al. 2010, Nagchowdhuri et al. 2012) have demonstrated versican's importance during joint formation. In the microarray, a recombinant adenoviral vector was used to up regulate the G1 domain of versican, and shRNA was used to silence versican. Significant changes in gene expression levels identified by the microarray and limited published information available regarding developmental specification led to the investigation of five matrix related genes (thrombospondin -3, discoidin domain receptor-2, sushi domain containing 5, matrix remodeling-associated 5, and matrilin 4).

In order to initiate studies into the potential roles played by these matrix-related genes and their regulation by versican, spatial expression of each gene was evaluated using *in situ* hybridization in sagittal chick wing sections. In most cases, with the exception of THBS3, gene expression patterns within developing chick joint regions had not been previously examined. We

hypothesized, due to the data from the microarray study, genes would show expression within or in close proximity to the elbow joint. *In situ* hybridization results revealed mRNA expression was present in the developing elbow joint, with interesting similarities and differences at sites of interzone expression. Furthermore, results gave insight into gene expression patterns within the surrounding tissues of the wing bud.

Thrombospondin-3 (THBS3) Expression Pattern and Implications For Joint Development

Thrombospondin-3 (THBS3) is an extracellular, calcium-binding glycoprotein with several functional domains. This allows for numerous binding partners including integrins, glycoaminoglycans (GAGs), and fibrillar collagens (Tan et al. 2006, Chen et al. 2005, Holden et al. 2001). THBS3 spatial localization within the developing elbow joint verified results previously reported in the chick model (Tucker et al. 1997). Like Tucker, *in situ* hybridization depicted mRNA expression localized along the presumptive articular regions with decreased detection present in the interzone and epiphyseal ends of the long bones. Findings also paralleled expression patterns in the murine model (Qabar et al. 1995) showing expression along the perichondrium.

The function of THBS3 in the developing elbow joint is unknown. Based on its structure, it may be able to interact with other THBS family members as well as independently of them (Bornstein et al 1993). This provides a possible explanation for the transcript localization found within the developing chick limb bud. THBS3 localization in chondrocytes and in the perichondrium, overlap expression of THBS1, and THBS2 in the avian model, respectively (Tucker et al. 1993, Tucker et al. 1997). In the dermis and muscle, THBS3 detection coincides with THBS 1 and 2 expression evaluated in the embryonic murine model (Iruela-Arisle et al. 1993, Tooney et al. 1998). Articular cartilage expression, however, is so far specific to THBS3,

indicating its possible independence and unique activities relative to other family members (Tucker et al. 1997).

Versican localization within the interzone illustrates temporal and spatial similarity to THBS3 expression. While interactions between THBS3 and versican have not been examined, several binding domains are thought to mediate possible interactions. All members of the thrombospondin family contain a high affinity heparin binding site at the N-terminus. This allows THBSs to bind negatively charged GAG chains like those found on versican and other sulfated proteoglycans (Tan et al. 2006). Furthermore, thrombospondins 1-4 have a laminin-G domain at their N-terminus, which is capable of interacting with link domains (Winnemoller et al 1992). THBS1 has proven to bind the link domain found within the G1 domain of versican (Kuznetsova et al. 2006). Although specific binding of THBS3 and versican has not been characterized, these domains implicate two possible ways interactions could occur during joint development. Binding at the G1 domain could also suggest that THBSs may interact with versican when the G1 fragment is proteolytically cleaved (Capehart 2010).

THBS3 knockouts in the mouse have shown postnatal accelerated endochondral ossification (Hankenson et al. 2005) and decreased columnar alignment of chondrocytes at the growth plate (Posey et al. 2008). However, prenatal skeletal patterning displayed no differences when compared to WT embryos. This conclusion could suggest an overlap in function within the THBS family, allowing members to compensate in another's absence. Although variations in gene expression have not been examined in the THBS3 knockout model, this hypothesis is supported by the observed overlap in expression patterns, and the conserved homology found amongst family members.

Discoidin Domain Receptor-2 (DDR2) Expression Pattern and Implications For Joint Development

Discoidin domain receptor-2 is a transmembrane, Receptor Tyrosine Kinase that is activated by collagen. Therefore, detecting its expression within several tissue types was not surprising. Collagen type-1 is the most abundant collagen within the body explaining DDR2 localization in the dermis and muscle, while collagen type-2 is found in hyaline cartilage and the interzone prior to cavitation. DDR2 has also been implicated in chondrocyte maturation giving insight into its expression within the long bones (Zhang et al. 2011), although its expression in developing joint tissues has not previously been documented.

Before the initiation of cavitation, cells within the interzone line up and flatten. This event also marks a change from type-2 collagen to type-1 collagen secretion (Craig et al. 1987). This could help to explain the transient differences in DDR2 transcript expression within the elbow and carpal joints. When activated by type-2 collagen, DDR2 promotes the activation of matrix metalloproteinase 13 (MMP13), which cleaves type-2 collagen (Xu et al. 2005). Furthermore, the later activation of DDR-2 by type-1 collagen, promotes MMP2 synthesis, which acts as a type-1 collagenase (Karagiannis et al. 2006). This pathway would presumably lead to changes in interzone structure in possible preparation for cavitation. While the mechanism for DDR2 is unknown, DDR-1 collagen activation triggers endocytosis of the receptor-ligand complex leading to lysosomal degradation or receptor recycling (Fu et al. 2013). The proximal-distal developmental gradient between elbow and carpal joint formation may explain why DDR2 is still highly concentrated within the carpal interzone at e9. Developmentally, the younger carpal joint will not reach cavitation at the same stage as the elbow, leading to possible differences in DDR-2 expression. This hypothesis is further supported

by the results from DDR2 HH34 (e8) *in situ* hybridization experiments. DDR2 transcripts in the younger elbow illustrated uniform expression throughout the articular and interzone regions much like the e9 carpal joint. The continued presence of DDR2 within the developing e9 elbow, once robust signal has left the interzone, is explained by the high concentration of type-2 collagen found within the developing articular cartilage.

MMP2 and MMP13 may also be implicated in further roles within the developing joint. Activated by DDR2, both molecules are known to cleave versican. If they are working to cleave versican as well as type-1 and 2 collagens, this could be a mechanism to promote the formation of the G1 fragment seen in the interzone during joint formation (Capehart 2010).

Strong expression of DDR2 transcripts in the developing limb extended beyond the joint regions and into the dermis and muscle. Collagen type-1, is present in both tissues. DDR-2 activation has been found to regulate fibroblast differentiation and migration, which occurs in both skeletal muscle and dermal tissue (Olaso et al. 2001). Furthermore, DDR-2 is known play a significant role in dermal wound healing. Its asymmetrical expression along the posterior side of the e9 limb bud however, is confounding. Transcript localization was present in the hind limb of the HH25 (e5) embryo, but showed no asymmetry. One explanation could be feather patterning within the dermis. Feather ontogenesis begins in the wing around stage HH39 (e13) (Meyer and Baumgartner 1998), with proliferation preferential to posterior feather buds (Chuong et al. 2000). While still several days away, interactions between the ectoderm, dermis, and underlying mesenchyme may be altering matrix structure in preparation for feather bud induction.

Sushi Domain Containing 5 (SUSD5) Expression Pattern and Implications For Joint Development

Very little is known regarding Sushi Domain Containing 5 (SUSD5) function or expression. Transcript localization illustrated similar patterning to DDR2 in the elbow and carpal joints. In the elbow transcripts were localized to the articular region with strong detection in the developing olecranon process of the ulna. Interestingly, versican has been localized in the developing limb in the same region and stage, and both proteins are capable of binding hyaluronan (HA) via link domains. Versican localization has not been examined extensively in the carpals, however the asymmetrical expression within the articular surface of the medial radius could be due to similar interactions. HA is known to be highly concentrated within the interzone of the developing joint leading to its possible interaction with SUSD5 in the carpal joint at e9. HA's potential interaction with SUSD5 would provide an alternative to its already well-established interaction with CD44. While CD44 has widespread expression, HH25 (e5.5) chick embryo *in situ* hybridization illustrated transcripts in the trunk, restricted to only to notochord. This limited expression pattern may suggest a specific necessary function during embryogenesis, perhaps separate from that of CD44. It's possible that HA could selectively bind to SUSD5 preceding its interaction with CD44, holding it prior to cavitation.

In addition to the link domain, SUSD5 contains a sushi domain, which is conserved in other adhesion proteins like fibulin-7 (de Vega et al. 2007). One explanation for its role within the developing joint could be to promote adhesion of the flattening cells that make up the articular surfaces during cavitation. Another explanation could be that it is acting as an inhibitor of cavitation by promoting cellular adhesion prior to HA/CD44 interaction. In the developing bone, adhesion properties could extend to the stacking of newly developed chondrocytes prior to hypertrophy. Adhesive properties necessary for muscle mass organization, growth and development could also explain its expression within skeletal muscle.

Matrix Remodeling- Associated 5 (MXRA5) Expression Pattern and Implications For Joint Development

MXRA5 is an extracellular matrix protein with very little characterization to date. It is capable of binding vascular endothelial growth factor (VEGF), implicating it in the numerous developmental pathways involving vascularization. Its localization within the developing chick limb is interesting due to its restriction within the interzone. One explanation could be that MXRA5 is acting to protect the synovial joint and articular surfaces from being inappropriately vascularized. By sequestering VEGF, the unbound form of the protein would be less concentrated in the avascular articular tissue and presumptive joint cavity. Another possibility could be that MXRA and VEGF bind preferentially prior to cavitation to stop vascularization within the interzone but release post cavitation. After cells migrate during cavitation, MXRA5 could release VEGF to facilitate vascularization of the synovial capsule. This is supported by the spatial expression differences in the developing elbow and carpal joints. Like DDR2 and SUSD5, localization within the elbow is retained to the articular surface, while carpal expression comprises the complete interzone.

The asymmetric patterning of MXRA5 in both elbow and carpal regions is interesting. Vascularization is occurring within tissues surrounding the joint, therefore MXRA localization could be an indirect measure of VEGF activity. Previously bound VEGF may be disassociating from MXRA5 to promote vascularization. This would help to explain its expression lateral to the presumptive joint in the muscle.

Matrilin-4 (MATN4) Expression Pattern and Implications For Joint Development

Matrilin 4 (MATN4) displayed very little signal within the developing elbow joint region, however weak expression of transcripts was detected along the articular surfaces of the

ulna and radius. Interestingly, these results paralleled later stage articular cartilage localization in the murine model (Klatt et al. 2002). While intensity differences were apparent, the timing of MATN4 gene expression could explain the low signal at (E9) compared to strong localization ex-utero. Expression along the perichondrium contrasted slightly with previous findings in chick model (Bandyopadhyay et al. 2008). In the present study, detection was found within the perichondrium rather than in subjacent chondrocytes. The weak signal expressed in both the articular surface and perichondrium could also question the binding efficiency of the designed probe. In the human gene there are four isoforms of MATN4. Currently in the chick genome only one variant exists within the NCBI database, however alternative splicing could explain a decrease in signal due to low affinity transcript binding.

The function of matrilin4 is currently unknown however, it contains 2 Von Willebrand factor A domains, conserved in integrins and several forms of collagen (Colombatti et al. 1993). Proteins containing this domain are thought to participate in cell adhesion, migration and patterning. Interestingly MATN4 also has 4 EGF domains, which is also conserved in versican. With similarities in gene expression along the articular surface, these proteins could interact with similar molecules such as collagens and integrins during joint formation. Microarray results from other labs have shown MATN4 expression within the dermis ex utero, however its dermal expression during embryonic development has not been characterized (Plager et al. 2007). The 4 EGF domains are conserved within a variety of ECM proteins including the Thrombospondin family members. Furthermore interactions between Thrombospondins and Matrilins have been confirmed (Mann, 2004). While these studies have not involved THBS3 and MATN4 specifically, expression patterns of both genes illustrate overlap and therefore present the possibility of protein interaction if mRNA translation occurs.

Summary

Versican is a large extracellular matrix protein integral for proper joint formation. It stands to reason due to its numerous binding domains and necessity in joint development, that versican manipulation could cause significant down-stream effects on gene expression levels as detected by the microarray. The goal of this study was to determine spatial expression patterns for THBS3, DDR2, SUSD5, MXRA5, and MATN4 whose expression was altered by experimental modulation of versican. This was done not only to characterize possible interactions with versican and other ECM molecules, but to also expand the limited published knowledge currently available regarding each of the five investigated. Results from the study indicated gene expression was present within the developing joint region for each of the five genes, and by examining previously published findings, several hypotheses were developed to detail how the genes could possibly interact with versican. While both function and mRNA translation are relatively unknown for most of the genes, the spatial localization of each gives further indication that the microarray was correct. Quantitative fold change data, along with gene expression localized to the developing joint, produces strong evidence that versican manipulation directly affects gene expression of each of the five genes examined. Results suggest that in addition to the molecular constituents previously identified in joint formation, there are several other factors involved, as well as the high likelihood of numerous cooperative interactions between them. As we move forward and continue examining several of these genes, our goal is to characterize mRNA and protein expression both quantitatively and qualitatively, at several developmental time points. This will not only verify mRNA transcripts are being translated, but it will also give a temporal timeline further characterizing proteins affected downstream of versican.

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Appendix A. Animal use protocol.



Animal Care and Use Committee

212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

January 9, 2012

Anthony Capehart, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. Capehart:

Your Animal Use Protocol entitled, "Versican G1 Domain and Hyaluronan Interaction in Synovial Joint Morphogenesis" (AUP #D269) was reviewed by this institution's Animal Care and Use Committee on 1/09/12. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

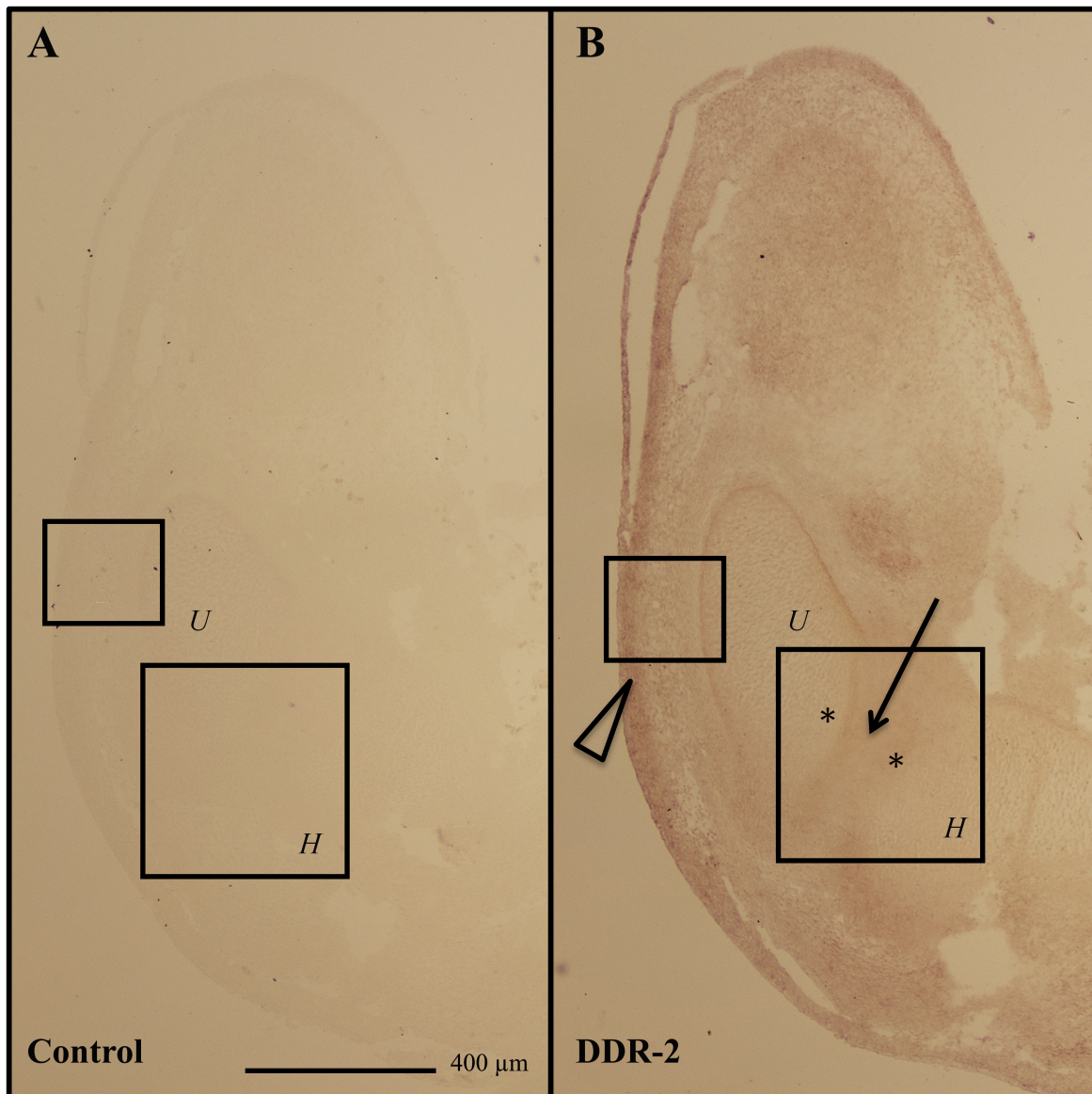
A handwritten signature in black ink, appearing to read 'S. E. Gordon'.

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

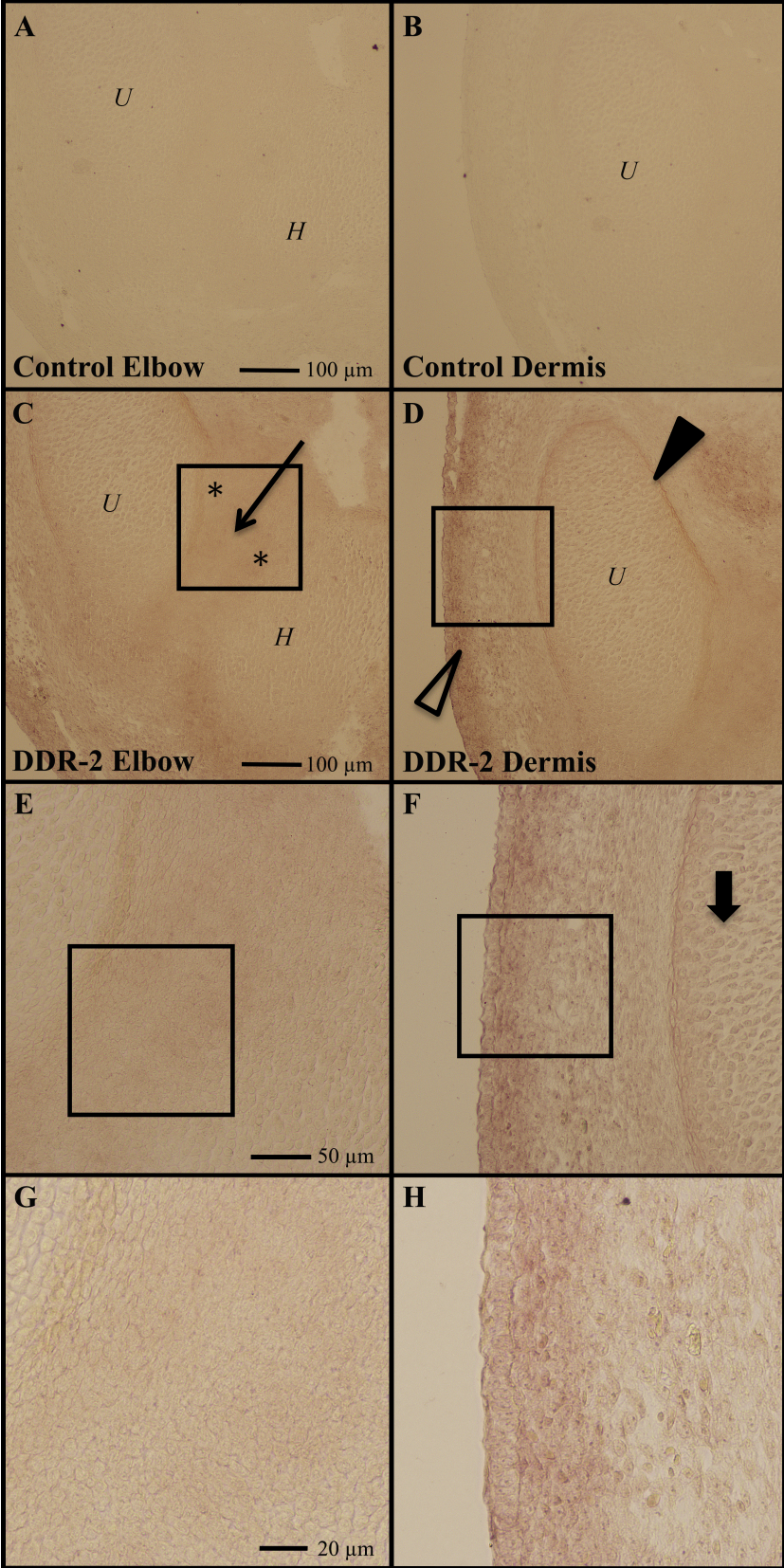
enclosure

Appendix B. Discoidin Domain Receptor- 2 (DDR2) *in situ* hybridization in sagittal sections of chick wing at HH34 (e8).



Appendix B. Discoidin Domain Receptor- 2 (DDR2) *in situ* hybridization in sagittal sections of chick wing at HH34 (e8). A: Little or no DDR2 expression present in control limb tissues hybridized with sense probe. B: DDR2 mRNA localization moderate in elbow extending throughout articular surface (*) and interzone (arrow). Prominent expression viewed in dermis (empty arrowhead) and perichondrium (filled arrowhead). Moderate expression also visualized in skeletal muscle (bold arrow). Regions containing substantial expression (boxed) are magnified in Appendix B. Scale bar in A= 400 μ m for A&B.

Appendix C. DDR2 HH34 (e8) areas of interest under increasing magnification.



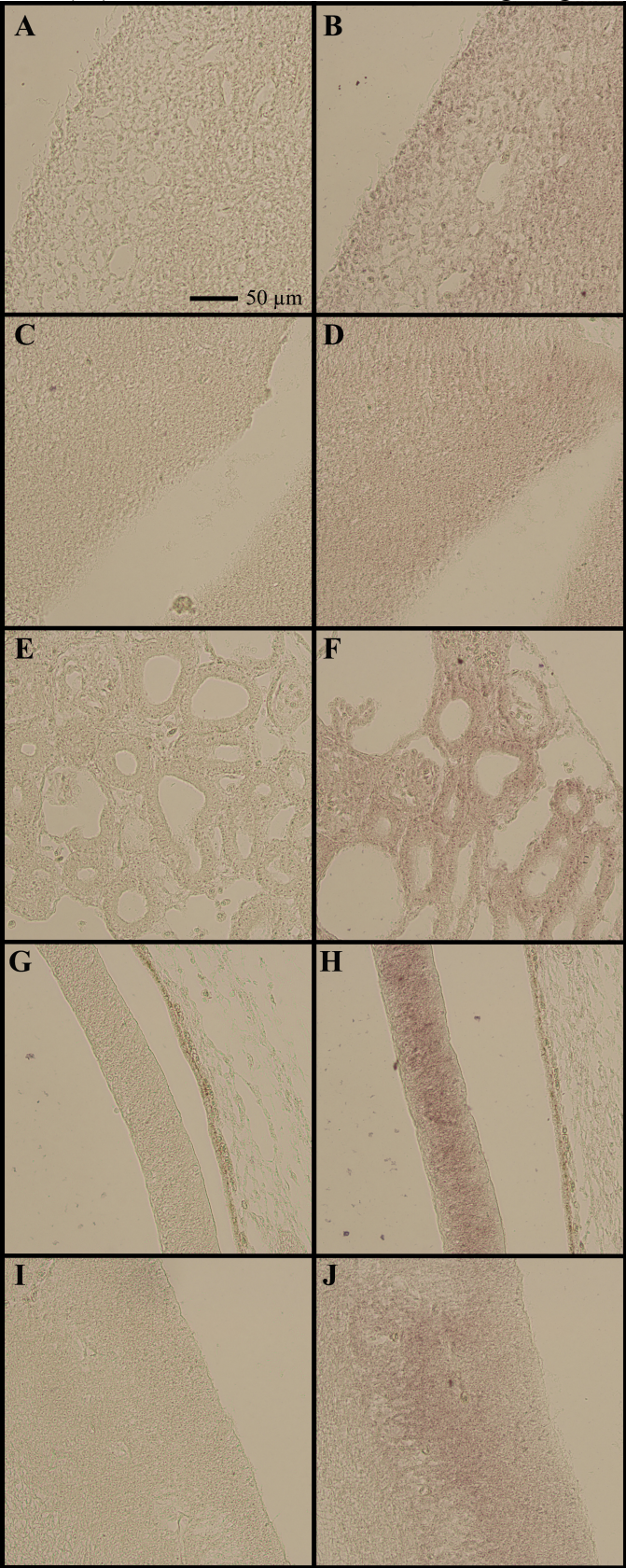
Appendix C. DDR2 HH34 (e8) areas of interest under increasing magnification. Elbow (left) and ulna (right) depict regions of DDR2 mRNA detection. Controls (A and B) show little to no specific localization with minor background. mRNA expression in the developing articular region (*) of humerus (H) and ulna (U) and within interzone (arrow) shows similarity in degree (C). Magnification of boxed region in (C) shows uniform expression throughout interzone region (bracket), decreasing in epiphyseal ends of humerus and ulna (E). (G) portrays further magnification and illustrates moderate intracellular localization. ulnar expression (D) portrays higher transcript localization in perichondrium (arrowhead) and dermis (open arrowhead). Magnification of (D) shows mild transcript localization in connective tissue deep to dermis (F), and in chondrocytes (bold arrow). Scale bar in A= 100 μ m for A-D. Scale bar in E= 50 μ m for E&F. Scale bar in G= 20 μ m for G&H.

Appendix D. *DDR2 in situ* hybridization in sagittal chick Embryo HH25 (e5.5).



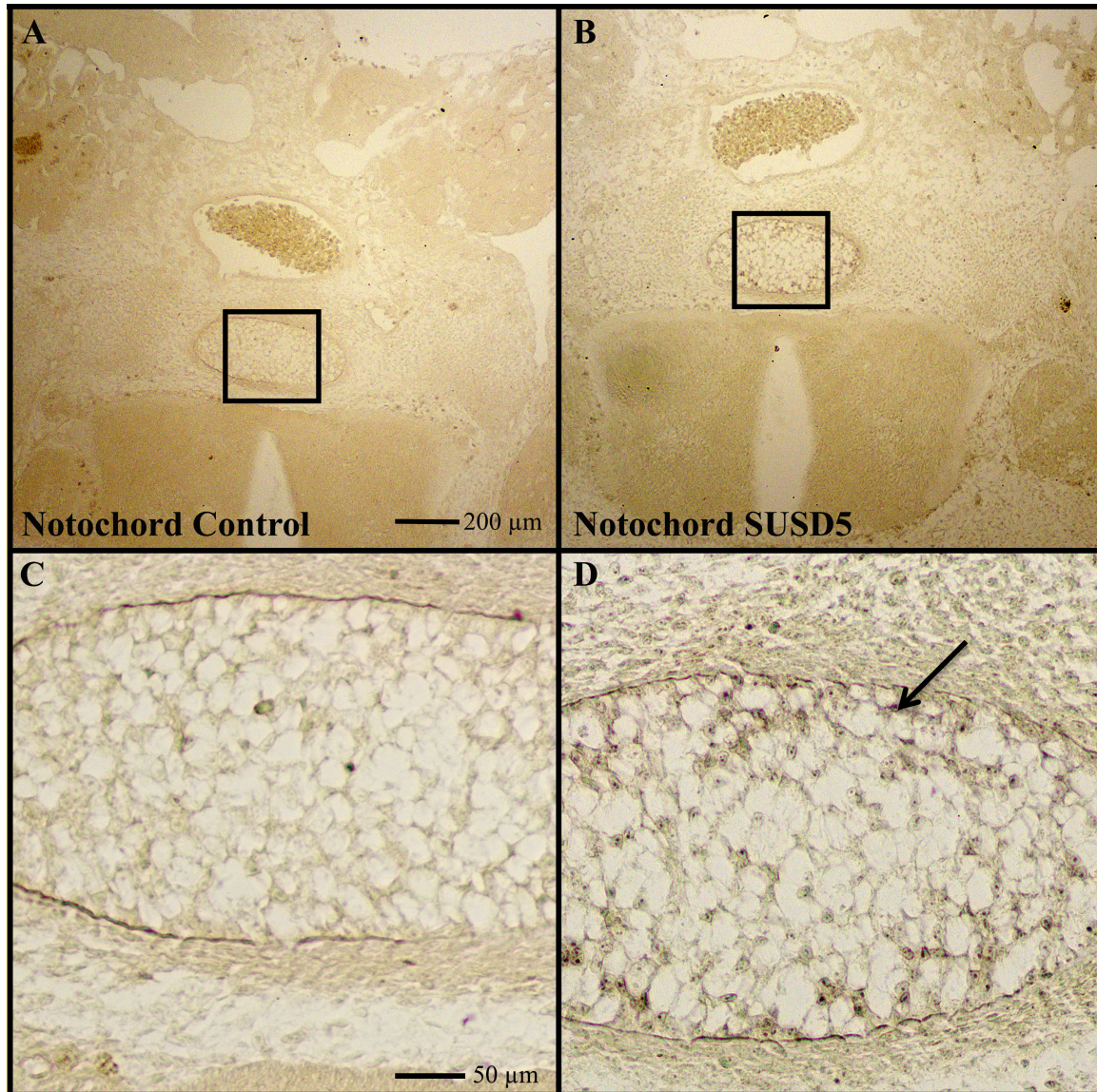
Appendix D. DDR2 *in situ* hybridization in sagittal chick Embryo HH25 (e5.5). A: little to no DDR2 specific expression present in control embryo with limited sense probe background. B: DDR2 in chick embryo shows transcript localization in several regions (boxes). Moderate expression in developing hind limb (arrow), neural tube (*), and mesonephros (open arrowhead). Higher localization in sensory retina (filled arrowhead) and developing brain vesicles (bold arrow). Regions containing expression (boxed) are magnified (Appendix D). Scale bar in A: 800 μm for A&B.

Appendix E. DDR2 HH25 (e5) areas of interest under increasing magnification.



Appendix E. DDR2 areas of interest under increasing magnification. Controls (left) show little to no specific DDR2 mRNA expression. Moderate transcription levels are visualized in the dermis of the hind limb (B), neural tube (D), and mesonephros (F). Stronger mRNA localization viewed in sensory retina (H) and developing brain vesicles (J). Scale bar= 50 μ m all images.

Appendix F. SUS D5 *is situ* hybridization in transverse sections of Notochord at HH25 (e5.5)



Appendix E. SUS D5 *in situ* hybridization in transverse sections of Notochord at HH25 (e5.5). Control, low magnification (A) and high magnification (C), show little to no SUS D5 background localization. Susd5 transcript localization is confined to the notochord (B). When boxed area is magnified, (D), SUS D5 expression is visualized along outer periphery of the notochord (Arrow) decreasing toward its center. Scale bar: 200 μm A&B and 50 μm C&D.