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Types of Co-existing Chronic Physical Conditions and Newly-diagnosed Depression, its Treatment and Economic Outcomes among Medicaid Beneficiaries with Type 2 Diabetes

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Evolution of a Disintegrin and Metalloproteinase Gene Family in Vertebrates

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**Thesis submitted to the
Eberly College of Arts and Sciences
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in partial fulfillment of the requirements
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**Master of Science
in
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ABSTRACT

Evolution of a Disintegrin and Metalloproteinase Gene Family in Vertebrates

Shashwati Bhattacharya

A disintegrin and metalloproteinases (ADAMs) are a family of transmembrane proteins which have diverse functions in various tissues. They play important roles in cellular and developmental processes and are present in various species. ADAMs are classified according to their expression in tissues into the somatic ADAMs (sADAMs) and the testicular ADAMs (tADAMs). The sADAMs are expressed in somatic tissues, and the testicular ADAMs (tADAMs) are expressed predominantly in the testis. The tADAMs are further classified into Group I and Group II, based on the absence or presence of introns. The mechanism of evolution of the tADAMs was unknown. Using bioinformatics tools, we performed an analysis of the evolution of ADAM genes in various vertebrates from fish to mammals. Our results show the duplication and loss of tADAMs in certain vertebrate species. In our phylogenetic analysis, all the tADAMs cluster together with ADAM9 and ADAM9-like, which we identified as a close paralog of ADAM9 in certain non-mammalian vertebrates, but are more distantly related to other sADAMs. Our synteny analysis shows that most Group II tADAMs lie next to the *Adam9* gene locus and hence likely arose from tandem duplication. On the other hand, Group I tADAMs are intronless, so they likely originated through retroposition. Therefore, we hypothesize that ADAM9/9-like and the tADAM loci are hotspots of gene duplication because many events of tandem duplication and retroposition have occurred in these regions. Some tADAMs were pseudogenized in certain species as a result of duplication events. The rapid duplication of ADAM genes resulted in diverse functions of ADAMs in various species and hence contributed to the evolution of species.

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1. INTRODUCTION

1.1. Background:

The “A disintegrin and metalloproteinase” (ADAM) family of proteins play important roles in cellular and developmental processes. The first two members of the ADAM family were identified as guinea pig sperm-surface proteins that were recognized by the monoclonal antibody PH-30 (Primakoff *et al.*, 1987). These sperm surface proteins from guinea pig were found to have a metalloproteinase-like and a disintegrin-like domain, and are related to the family of snake venom protein domains (Blobel *et al.*, 1992; Wolfsberg *et al.*, 1993). These proteins were therefore named ADAM for containing “A Disintegrin” and “A Metalloproteinase” domain (Wolfsberg *et al.*, 1995).

ADAMs belong to the metzincin superfamily of proteases (Bode *et al.*, 1996). This superfamily of proteases have a zinc binding consensus sequence HExxHxxGxxH that is present in all proteolytically active members (Stocker *et al.*, 1995). Zinc-dependent proteases are classified into gluzincin, metzincin, inuzincin, carboxypeptidase, and DD carboxypeptidase subgroups according to the primary structure of their catalytic sites (Hooper NM. 1994). The ADAMs belong to the metzincin subgroup which is subdivided into serralysin, astacin, matrixin, and adamalysin (Stocker *et al.*, 1995). The serralysins are a family of proteolytic enzymes that are secreted by various bacteria of genus *Serratia* (Nakahama *et al.*, 1986). Astacins are proteases which are found in wide range of organisms and function in processing proteins (Bond *et al.*, 1995). The matrixins comprise the matrix metalloproteinases (MMPs). Adamalysins, including ADAMs, have a metalloproteinase domain and a unique integrin receptor-binding disintegrin domain.

ADAMs are mainly multi-domain type I transmembrane proteins. Some ADAMs can be present in a soluble form because of alternative splicing or protease-mediated cleavage. ADAMs are present in invertebrate species such as nematode *Caenorhabditis elegans* and all vertebrates. Adam-like sequences have been found in the yeast *Saccharomyces pombe* (Edwards *et al.*, 2008).

ADAMs are involved in a variety of functions. In mammals, many of the ADAMs are expressed in somatic tissues, and are known to have a role in cell fate determination in the central nervous system, cell migration, as well as axon and muscle development (Primakoff *et al.*, 2000). Functions of ADAMs vary from neural crest development in frogs and chicken, adipogenesis and myogenesis in mice, to the fusion of sperm and egg in mice (Rooke *et al.* 1996; Kurisaki *et al.*, 2003; Cousin *et al.*, 2011; Schiffmacher *et al.*, 2014). The main functions of ADAMs are proteolysis, adhesion and intercellular signaling. Biological functions of many ADAMs are still unknown. ADAMs have been implicated in many diseases, including cancers, immune diseases and neurodegenerative diseases (Edwards *et al.*, 2008). For example, ADAM9 is upregulated in many cancers and also spreads colon cancer cells by binding to integrins $\alpha 6\beta 4$ and $\alpha 2\beta 1$ (Edwards *et al.*, 2008). Some ADAMs stimulate cell proliferation via EGFR receptor activation and cause growth of malignant tumors (Borrito *et al.*, 2003; Kenny *et al.*, 2007). On the other hand, ADAMs can also be used for therapeutics. For example, ADAM10 plays an important role in Alzheimer's disease by participating in non-amyloidogenic processing of amyloid-beta precursor protein (APP) and hence helps in alleviating the progress of the disease (Cong *et al.*, 2009). Therefore, therapies can be developed by either promoting or inhibiting ADAM activities depending on the disease.

1.2. Structure of ADAMs:

The basic structure of ADAMs consists of a signal peptide, a prodomain (PRO), a metalloproteinase domain (MP), a disintegrin domain (DIS), a cysteine-rich domain (CRD), a transmembrane region (TM) and a cytoplasmic tail (Figure 1). A signal peptide at the N-terminus of an ADAM directs it into the secretory pathway and the prodomain functions as a chaperone to facilitate the proper folding and secretion of ADAMs (Roghani *et al.*, 1999). The prodomain also prevents self-cleavage by binding and inhibiting the MP domain. A “cysteine switch” in the prodomain of some ADAMs function to regulate the MP domain, and hence regulate proteolysis (Zhu *et al.*, 1999, Brachvogel *et al.*, 2003). Proprotein convertases cleave the prodomain in the Golgi, resulting in activation of ADAM proteases (Schlondorff *et al.*, 1999). The MP domain in proteolytically active ADAMs contains a consensus sequence HExxHxxGxxH, which binds the catalytic zinc ion. The zinc ion is necessary for hydrolytic processing of protein substrates. A ‘Met turn’, i.e., a methionine residue C-terminal to this consensus sequence, stabilizes the zinc ion and maintains the structure of the active site (Seals *et al.*, 2003). About half of the ADAMs lack this consensus sequence in the MP domain, and hence lack the protease activity (Brocker *et al.*, 2009). The DIS domain has a disintegrin loop, which is responsible for the interaction with integrins (White, 2003). ADAMs, except for ADAM10 and ADAM17, have a Rx6DEVF sequence in their DIS domain which interacts with the $\alpha 9\beta 1$ integrin (Eto *et al.*, 2002). The CRD of ADAM12 is involved in cell-matrix adhesion by acting as a ligand for syndecan (a cell adhesion molecule) (Seals *et al.*, 2003). The CRD of *Xenopus* ADAM13 has been shown to interact with the glycoprotein fibronectin (Gaultier *et al.*, 2002). The cytoplasmic tail varies in length among various ADAMs, and may contain binding sites for different proteins (Cho, 2012). Several ADAMs have one or more PXXP sites for binding to SH3 motif-containing proteins, which interact with various intracellular proteins. For example, the cytoplasmic tail of ADAM12

interacts with alpha-actinin2, a cytoskeletal protein which binds actin (Cao *et al.*, 2001). Through interaction with cellular proteins, the cytoplasmic tail may be involved in intracellular signaling.

1.3. Functions of ADAMs:

Some somatic ADAMs play important roles in cell fate determination, cell migration, and axon and muscle development (Primakoff *et al.*, 2000). For example, ADAM8 is a transmembrane glycoprotein expressed in immune cells, and contributes to neuron-glia interaction in the central nervous system (Schlomann *et al.*, 2000). ADAM12-deficient mice have defects in the formation of adipose tissue and myogenesis (Kurisaki *et al.*, 2003). Some ADAMs cleave EGFR ligands to activate the EGFR pathway which is responsible for cell migration, differentiation and tissue homeostasis (Liebmann, 2011, Wong, 2003). For example, ADAM17 is required for the shedding of EGFR ligands TGF α , HB-EGF, and amphiregulin, and ADAM10 is involved in cleaving two EGFR ligands EGF and betacellulin (Sahin *et al.*, 2004). ADAM10 also processes both the N- and E-cadherins, which are involved in physiological functions such as neurulation, somitogenesis and tissue morphogenesis (Maretzky *et al.*, 2005, Reiss *et al.*, 2005). ADAM proteases also cleave cytokines and cytokine receptors, which play important roles in immune responses (Edwards *et al.*, 2008, Jin *et al.*, 2004, Kurzrock 2001). For example, ADAM17 cleaves TNF- α (tumor necrosis factor alpha), which is a proinflammatory cytokine and regulates immune cells (Black 2002). Finally, ADAMs are involved in a process called regulated intramembrane proteolysis (RIP). RIP is the cleaving of an integral membrane protein to release a soluble protein which is involved in cellular functions like apoptosis, cell proliferation, etc. (Edwards *et al.*, 2008). Activation of the Notch receptor is a RIP process and ADAMs participate in this process. When the Notch receptor binds to the Notch ligand, it undergoes a series of proteolytic cleavages, which release the Notch intracellular domain. Ligand activates Notch and ADAM10 cleaves the extracellular S2 proteolytic site of Notch. This cleavage generates a

membrane-bound “stub” of Notch which is cleaved further at S3 and S4 sites and releases the Notch intracellular domain that translocates to the nucleus and acts as a transcription regulator (Brou *et al.*, 2000).

1.4. Classification of tADAMs:

More than half of the ADAM proteases are predominantly expressed in the male testis. Cho (2012) divided the mouse reproductive ADAMs into three groups: (I) intronless tADAMs, (II) tADAMs with multiple exons and introns, and (III) ADAM7 and ADAM28. In mice, ADAM7 and ADAM28 do not show expression in the testes, but are expressed in the epididymis (Kim *et al.*, 2006). Phylogenetic analyses indicate that ADAM7 and ADAM28 do not cluster together with tADAMs (Figure 2). Group III, which consists of ADAM7 and ADAM28, was removed from the list of tADAMs based on these observations (Table 1, with Group III removed). But we keep the other two groups of tADAMs, i.e. Group I and Group II, based on their clustering in the phylogenetic tree (Figure 2).

Group I has 11 *tAdam* genes which lack introns in their coding sequences (Kim *et al.*, 2006). ADAMs 4, 6, 24, 25, and 26 are exclusively expressed in germ cells of the mouse testis.

Group II tADAMs are predominantly expressed in germ cells of the testes. ADAMs in Group II lack active protease activity since they lack the consensus sequence of HEXxHxxGxxH (Cho, 2012). There is evidence that some Group II tADAMs are also expressed in somatic tissues (Murase *et al.*, 2008).

1.5. Key roles of ADAMs in fertilization:

TADAMs play key roles in fertilization, which is initiated by the adhesion of sperm and ovum. In mice, ADAM1, along with ADAM2 and ADAM3, are responsible for adhesion of the sperm to the

zona pellucida, which is a glycoprotein membrane of the ovum (Evans, 2001). ADAM1, ADAM2 and ADAM3 are essential for sperm-egg fusion (Cerretti *et al.*, 1999). ADAM1 and ADAM2 form a heterodimeric complex on the mature sperm (Primakoff *et al.*, 1987; Blobel *et al.*, 1992, Wolfsberg *et al.*, 1995, Evans, 2002). ADAM2 and ADAM3 form a complex that helps the sperm to enter the oviduct *in vivo*, and promote sperm–egg interactions *in vitro* in mice (Cho, 2012). Trimeric complexes, such as ADAM2/ADAM3/ADAM4, ADAM2/ADAM3/ADAM5, and ADAM2/ADAM3/ADAM6, are formed on matured sperm, and are necessary for successful fertilization (Han *et al.*, 2009). ADAM18 is proteolytically processed by cleaving both the pro- and metalloproteinase-like domains and retaining the disintegrin-like domain during epididymal transit to yield mature proteins on matured sperm (Frayne *et al.*, 1998). ADAM21 is expressed in testicular somatic cells as well as germ cells (Yi *et al.*, 2010). The *Adam21* gene produces two types of transcripts. One is expressed in germ cells of the testis and has exon1 and exon2, while the other is expressed in testicular somatic cells and only has exon2 (Yi *et al.*, 2010). It is unclear whether ADAM21 is involved in fertilization or spermatogenesis. ADAM24 is localized on the mature sperm acrosome after its prodomain has been removed. The cytoplasmic tail of ADAM24 acts as a substrate for protein kinase C (PKC). After activation of ADAM24 by PKC, the sperm penetrates the zona pellucida (Zhu *et al.*, 2001). In *in vitro* assays, *Adam24*-knockout mice displayed reduced fertility with polyspermic fertilization, which can lead to abnormal embryonic development (Zhu *et al.*, 2009). ADAM25 and 26 (also called testase2 and testase3) are only found to be expressed in mouse spermatogenic process (Zhu *et al.*, 1999, Brachvogel *et al.*, 2003). Previous reports suggest that ADAM27 is expressed in the pachytene stage of spermatocytes of mice. *Adam32* gene expression begins during meiotic prophase in spermatocytes (Choi *et al.*, 2003). All of these studies on the reproductive ADAMs were performed on rodents. Since some of these genes are pseudogenes in the human genome, it is possible that functions of these tADAMs are species-specific.

1.6. Objectives:

The objective was to understand the mechanism of evolution of tADAMs. We wanted to find whether there are tADAMs in non-placental mammals and in non-mammalian vertebrates.

ADAM9 is a sADAM which is widely expressed and highly conserved among all vertebrates that clusters along with the tADAMs in the phylogenetic analyses (Figure 2). We hypothesized that all the tADAMs originated from a common ancestor shared with ADAM9. With the help of phylogenetic analyses and syntenic analyses, we wanted to explore the evolutionary origin of *tAdams*.

1.7. Significance of the study:

The study on the evolution of reproductive ADAMs provided key insights into the functions of these proteins and the actual mechanisms of gene duplication. ADAMs are critical for fertilization and could be helpful in developing new methods of *in vitro* fertilization.

2. MATERIALS AND METHODS

2.1. Identification of *Adam* genes in vertebrate species tested (i.e. opossum, chick, lizard, and frog):

The sequences of annotated human and mouse ADAMs were used to identify *Adam* genes from other species using the BLAST program from NCBI. With human tADAM as queries, the NCBI,

protein

BLAST

(ProteinBLAST[http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome]) was used to search against the species genome assembly. The representative species chosen were opossum, chick, lizard, and frog; other vertebrates such as rat, rabbit, pig, dog, Tasmanian devil, platypus, zebra finch, flycatcher, coelacanth, Japanese medaka, zebrafish, spotted gar and elephant shark were also included so that there was a representative organism from each vertebrate phylum. Three types of analyses i.e. phylogeny, synteny, and conservation of intron-exon boundaries were performed to identify the orthologs of predicted human and mouse ADAMs. The most appropriate BLAST hit i.e. the most significant match between sequences was selected. The lowest and closest to zero E-value is the most significant BLAST hit. The BLAST hit with E-value zero was selected, and the UCSC genome browser BLAT was used to search and identify the intron-exon boundaries in the *tAdam* genomic sequence (BLAT: <https://genome.ucsc.edu/cgi-bin/hgBlat?command=start>). Tissue expression patterns of specific *Adam* genes were obtained from the NCBI's UniGene website.

2.2. Phylogenetic analyses:

For phylogenetic analyses, amino acid sequences of ADAM from various species (Table 2) were aligned using default MUSCLE (MULTiple Sequence Comparison by Log Expectation) alignment parameters, and all positions containing gaps were eliminated (Edgar, 2004). Phylogenetic trees, including Bayesian, neighbor joining, and maximum likelihood trees, were constructed to investigate the evolutionary relationship among the ADAMs as described below. Three types of tree building methods were used to see if the clustering of the clades are consistent when the phylogenetic trees are built using different tree building methods.

The Bayesian Markov Chain Monte Carlo analyses were conducted with MrBayes version 3.2.2 (Ronquist *et al.*, 2003). This method is based on the posterior probability of the tree. The

posterior probability is the probability that the tree is correct and the evolutionary model used in the analyses is assumed to be correct. The bases of Bayesian inference is the Bayes's theorem:

$$\text{Pr}[\text{Tree} \mid \text{Data}] = \frac{\text{Pr}[\text{Data} \mid \text{Tree}] \times \text{Pr}[\text{Tree}]}{\text{Pr}[\text{Data}]}$$

This equation is used to calculate the posterior probability distribution on trees ($\text{Pr}[\text{Tree} \mid \text{Data}]$) by combining prior probability of a phylogeny ($\text{Pr}[\text{Tree}]$) with the likelihood ($\text{Pr}[\text{Data} \mid \text{Tree}]$). This suggests that the tree with higher posterior probability will be the favorable data for interpreting the phylogeny (Huelsenbeck *et al.*, 2001). There are a number of numerical methods to estimate the posterior probability, the most commonly used is the Markov chain Monte Carlo (MCMC) method. There are two steps in the MCMC. First, a new tree is made randomly from the current tree, then either this tree is accepted or rejected based on a probability described by Metropolis *et al.* (1953) and Hastings (1970). This is repeated several times. MrModeltest 2.3 compares 24 models of nucleotide substitution. The best-fit model was selected as recommended by the Akaike Information Criterion (AIC) in MrModeltest version 2.3 in the Bayesian analysis (Nylander, 2004). A model selection is the comparison of all models using AIC (Akaike, 1974). Each analysis was run for 3,000,000 generations with six Markov chains to achieve a mean standard deviation less than 0.01. Posterior probabilities (PP) were calculated, and the model parameters were stabilized at 2,400,000 generations. Trees were sampled every 100 iterations. The final tree was visualized with FigTree v4.1.0 (FigTree: <http://tree.bio.ed.ac.uk/software/figtree/>) (Bahudhanapati *et al.*, 2015).

Neighbor joining and maximum likelihood trees were generated by using the algorithms included in the MEGA 6.0 software. The neighbor joining tree is a data clustering algorithm based on similarity and distance matrix. The bootstrap consensus trees were inferred from 1000 replicates using the neighbor joining algorithm. The bootstrap value is the confidence

value added to each internal node of the tree. This represents the number of times that the branch clustering occurs in the replicate trees when they are resampled. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site (Tamura *et al.*, 2003). The evolutionary history was also inferred by using the maximum likelihood method based on the JTT matrix-based model (Zuckerkandl *et al.*, 1965). In this method, the tree that has the highest probability of generating the observed data is selected. JTT is an amino acid replacement model used in maximum likelihood to find the probabilities of change on tree branches. Several studies showed that JTT model is useful for the analysis of evolutionary history of membrane proteins. Since ADAMs are transmembrane proteins so we used the JTT model (Jones *et al.*, 1994). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees were obtained by applying the neighbor joining and BioNJ algorithms to a matrix of pairwise distances using the JTT model, and then selecting the topology with superior log likelihood value. JTT matrices used in this program MEGA6.0 were obtained from the source code of PHYLIP version 3.6 (Felsenstein *et al.*, 1993-2001). All positions containing gaps and missing data were eliminated (Bahudhanapati *et al.*, 2015).

2.3. Syntenic analyses:

For syntenic analyses, an individual *Adam* gene was entered into the search box of Genomicus (Louis *et al.*, 2013), to find the position of tADAMs with respect to other genes for all the species mentioned above. Metazome (Jones *et al.*, 1992) and the NCBI Map Viewer was also used to identify surrounding loci that are evolutionarily conserved. Data from these websites were analyzed to generate the syntenic diagrams (Bahudhanapati *et al.*, 2015).

2.4. Identification of retroposition event among the *Adam* genes:

Using the UCSC Genome Browser BLAT search, potential retroposed *Adam* genes were identified as *Adam* genes with no or few (≤ 2) introns. ADAM protein sequences were obtained from NCBI, and the sequence was entered in the search bar of UCSC Genome Browser and the splice sites were determined (Bahudhanapati *et al.*, 2015).

3. RESULTS

3.1. *TAdams* in various animal species:

Four *Adam* genes have been identified in the Ciona genome and twenty-two in zebrafish, but *tAdam* has not been identified in these non-mammalian species (Huxley-Jones *et al.*, 2007). *TAdams* had only been identified in eutherians, so we started looking for *tAdams* in non-eutherian vertebrates. We performed a search for *tAdam* genes in various vertebrate species, including frog (*Xenopus tropicalis*), lizard (*Anolis carolinensis*), chick (*Gallus gallus*), opossum (*Monodelphis domestica*), mouse (*Mus musculus*) and human (*Homo sapiens*) (Table 2), to understand the mechanism of evolution and the origin of *tAdams*. We found five *tAdams* in chick, nine in lizard and nine in opossum.

3.2. Story of ADAM9 and ADAM9-like:

ADAM9 is overexpressed in many human carcinomas, and plays an important role in tumorigenesis and angiogenesis. ADAM9 cleaves molecules such as EGF, EphB4, Flk-1, CD40, etc. The cleaving of these molecules plays important roles in tumorigenesis and angiogenesis (Peduto, 2009). *Adam9* knockout mice show retinal degeneration gradually with

age, but do not show any apparent developmental defects. Similarly, null mutations in ADAM9 result in cone-rod dystrophy in humans (Parry *et al.*, 2009). We could not identify *Adam9* in invertebrates like *Drosophila* and *Ciona*, but found the *Adam9* gene in teleosts as well as spotted gar. The syntenic locations of the nearby genes of *Adam9* in teleosts are rearranged relative to the position of *Adam9* and its nearby genes in spotted gar, amphibians and higher vertebrates (Figure 3).

We identified an *Adam9*-like gene in fish, amphibian, reptilian and avian genomes (Figure 4). We could not identify this gene in any mammals; hence it may have been lost in mammals (Figure 4). The protein sequences of ADAM9-like from various species are more similar to ADAM9 than to other somatic ADAMs. This suggests that ADAMs 9 and 9-like share a common ancestor. *Adam9* and *Adam9*-like are absent in the jawless lampreys, but have been identified in the cartilaginous fish elephant shark, one of the earliest vertebrate species. We were unable to identify which of these ADAMs evolved first because of the lack of genomic data available, but it can be speculated that both ADAM9 and ADAM9-like evolved early during vertebrate evolution.

3.3. Group I and Group II tADAMs cluster with ADAMs 9 and 9-like forming a subfamily:

Through an extensive search, we identified Group I and Group II *tAdams* in *Anolis*, chicken and opossum. Both Group I and Group II tADAMs of *Anolis*, chicken, opossum, mouse and human form clusters with high confidence with ADAMs 9 and 9-like in phylogenetic trees (Figure 2). In *Xenopus tropicalis* and fish genomes, we could not identify any definitive Group I or Group II tADAM, although ADAM16 of *Xenopus laevis* has been reported to be expressed predominantly in testis (Shilling *et al.*, 1997) and clusters with ADAM9 and ADAM9-like in phylogenetic trees

(Figure 2A). From the study, it can be said that Group I and Group II tADAMs and both the ADAM9 and ADAM9-like belong to the same ADAM subfamily. Since ADAM9 and ADAM9-like are found in all non-mammalian vertebrates including fishes and amphibians, both ADAM9 and 9-like probably emerged earlier than the tADAMs during vertebrate evolution, suggesting that Group I and Group II tADAMs evolved from ancient ADAM9 and/or 9-like.

In the previous study by Cho (2012), *Adams 7* and *28* were classified under the *tAdams* as “reproductive” *Adams*. Cho classified the *tAdams* into three groups which we have mentioned earlier. The Group III consists of *Adam 7* and *28*, and was called reproductive *Adams* because *Adam7* is predominantly expressed in the mouse epididymis (Cho, 2012). However, exploring the UniGene database, we found that human *Adam28* is expressed in a wide variety of somatic tissues (data not shown). The phylogenetic analyses strongly indicate that ADAM 7 and 28 do not cluster with the tADAMs in the phylogenetic tree. This evidence suggests that they not belong to the subfamily that includes ADAMs 9, 9-like and tADAMs. Group III, which consists of ADAM 7 and 28 based on Cho’s classification of “reproductive” ADAMs was removed and excluded from subsequent analyses.

3.4. Evolution of Group II *tAdam* genes:

We carried out a syntenic comparison of Group II *tAdams* for all representative amniote species and found that Group II *tAdams* cluster together in a genomic region next to *Adam9* (Figure 4). We found two Group II *tAdam* genes in the lizard (*Anolis carolinensis*) and chicken (*Gallus gallus*) genomes. Three Group II *tAdams* were found in opossum, four in mouse, and five in the human genome. It is only in the mouse and rat genomes that the *Adam2* gene is on a different chromosome (chromosomes 14 and 15, respectively), but all the other Group II *tAdams* are localized near *Adam9* (Figure 3). No *Adam* gene was found near the locus of *Adam9* in the *X. tropicalis*, coelacanth, or zebrafish genome (Figure 3). These results suggest that tandem

duplication i.e. the event of copying the gene adjacent to the original copy; gave rise to Group II *tAdam* genes, and that an ancient *Adam9* was the founder gene of the very first Group II *tAdam* during evolution. Unequal crossing over during homologous recombination between paralogous sequences gives rise to tandemly duplicated genes. Deletion and inversion of genes are also the result of unequal crossing over in homologous recombination. The organization of Group II *tAdam* genes in an array on the same genomic locus is mainly a result of tandem duplication. Gene duplication also gives rise to pseudogenes. In the human genome, among the Group II *tAdams* we found that *Adam3* and *Adam5* are pseudogenes (HGNC: <http://www.genenames.org/>).

3.5. Evolution of Group I *tAdam* genes: evidence for retroposition:

A characteristic of Group I *tAdams* is that they contain none or very few introns. Retroposition is a mechanism in which a messenger RNA is reverse transcribed into cDNA with the help of reverse transcriptase, then the newly transcribed intronless DNA sequence from the mRNA gets inserted into the genome. Since the events in retroposition lead to intronless genes, it has been speculated that Group I *tAdams* are derived from retroposition. These Group I *tAdams* have lost the signature of retroposed genes i.e., the poly A tail because they evolved a long time ago. In our phylogenetic study, Group I *tADAMs* cluster with *ADAM9* and 9-like and Group II *tADAMs*, so it can be speculated that Group I *tADAMs* share a common ancestor with Group II *tADAM*, *ADAM9* and 9-like. Thus, the parental gene that gave rise to a specific Group I *tAdam* could be *Adam9*, 9-like, a Group II *tAdam*, or another Group I *tAdam* (Bahudhanapati *et al.*, 2015).

3.6. A recent event of retroposition*:

[*Note: This is Dr. Hari Bahudhanapti's work.]

We started looking for retroposed genes in the chicken, lizard, opossum, mouse and human that were derived from the ADAM9/9-like/tADAM subfamily in which the characteristic features of retroposition may still be well preserved.

In the opossum genome, we identified an additional copy of *Adam9* that has only one intron, which we named “*Adam9b*” (Figure 5). This gene is ~99% identical to another copy, i.e. *Adam9a*, as per their nucleotide sequences. The *Adam9a* gene has 24 introns and conserved exon/intron boundaries with *Adam9* in other vertebrate species (data not shown), hence is the opossum orthologue of *Adam9*. Since *Adam9b* has only one intron and a very high sequence homology to *Adam9a*, we believe that *Adam9b* was likely retroposed. We also found a polyA tail in *Adam9b* (Figure 5) which supports the recent retroposition of *Adam9b*. In the event of retroposition, the cDNA copy is inserted randomly and the non-LTR retroposons like LINE has an adenosine-rich region at their 3'ends. Since *Adam9b* has a polyA tail and is almost intronless, it indicates that it is a retroposed gene. We could not identify any other retroposed copy of other *Adams* in the opossum genome. This suggests that *Adam9* may be a preferred target for retroposition.

3.7. Tandem duplication of Group I *tAdams*:

The Group I *tAdams* were found in clusters in some species. An example is *Adam1* duplication. Genes near the *Adam1* locus are conserved from frogs to humans, although *Adam1* was not found in *Xenopus tropicalis* in this genomic region. This region in mammals and chicks is flanked by *Tmem116*, *Erp29* on one side and *Mapkapk5*, *Aldh2* on the other side. *Anolis*

carolinensis has one *Adam1*, while the Chinese turtle has a duplicated *Adam1* (Figure 6). Two copies of *Adam1* are present in the chicken, opossum, mouse and human genome, suggesting tandem duplication. However, no orthology was found between mammalian and chicken *Adam1a* and *1b*. So we hypothesize that either *Adam1* has been duplicated separately in chicken and mammals, or before the divergence of mammals and one copy of it was lost in certain species such as *Anolis carolinensis* (Figure 6) (Bahudhanapati *et al.*, 2015).

4. Discussion:

Gene duplication arises from unequal crossing over, retroposition, or chromosomal (or genome) duplication. These three different processes generate three different outcomes. Unequal crossing over results in tandem duplication, i.e. duplicated genes are present on the same chromosome close to each other. Position of crossing over determines whether the duplicated region has an entire gene or not. Retroposition can be characterized by loss of introns, loss of regulatory sequences, presence of polyA tails, or presence of flanking short direct repeats.

Pseudogenization or the loss of function of the duplicated gene is a common feature of gene duplication. We found 11 *tAdam* pseudogenes, which include both Group I and Group II *tAdam* genes (HGNC: <http://www.genenames.org>). These Group I *tAdams* which have probably originated from the event of retroposition, have a pre-mature stop codon. They are expressed and produce a truncated protein which is non-functional. So these Group I *tAdams* are not pseudogenes because of lack of transcription, which is commonly seen in retroposed genes since they do not carry the regulatory elements essential for transcription (Kaessmann *et al.*, 2009). In retroposition, there is always a chance of insertion of the cDNA into a genomic location that is downstream of a promoter sequence; hence, causes the retroposed genes to get expressed (Vinckenbosch *et al.*, 2006). Interestingly, retroposed genes and duplicated genes

are commonly found to be expressed in the testes (Kaessmann *et al.*, 2009, Vinckenbosch *et al.*, 2006).

Gene duplication can work in two ways that can lead to evolution. One way is that the duplicated gene copy is producing proteins for a new function and the process is called neofunctionalization, or the multiple functions of the ancestral gene are divided into the newly duplicated genes and it is called subfunctionalization. The process of neofunctionalization will sustain if the new functions are advantageous. These duplicated *tAdam* genes which are predominantly expressed in testis, may also expand their expression to other tissues. It was found from the UniGene database, that Group II *tAdams* of mouse and human have transcripts expressed in some somatic tissues (Table 3).

5. CONCLUSION

In this study, we identified homologues of mouse and human tADAMs in the non-mammalian vertebrates chicken, lizard, and non-placental mammals such as the opossum. From our phylogenetic analyses, we found that the tADAMs cluster with ADAM9 as well as ADAM9-like, which we identified only in the non-mammalian vertebrates. It can be suggested that all the tADAMs have originated from an ancient somatic ADAM9. TADAMs were classified into three groups. We proposed to remove Group III, consisting of ADAM7 and ADAM28 from the classification because in our phylogenetic analyses Group III did not cluster with the tADAMs. We infer from our phylogenetic analyses that ADAMs 7 and 28 do not belong to the subfamily that includes ADAMs 9, 9-like and tADAMs.

The Group I *tAdams* are intronless, and are likely derived from retroposed transcripts, and some have undergone further tandem duplication in some vertebrates. One example of tandem duplication in Group I *tAdams* is the clustering of all the testase genes (nine Group I *tAdams*) in

a single chromosomal region (Bahudhanapati *et al.*, 2015). Phylogenetic analyses indicate that the Group I *tAdams* share a common ancestor with the Group II *tAdams*, *Adam9* and *9-like*. Thus, the parental gene that gave rise to a specific Group I *tAdam* could be *Adam9*, *9-like*, a Group II *tAdam*, or another Group I *tAdam*. Group II *tAdams* cluster together in a genomic region next to *Adam9* suggesting that tandem duplication from an ancient *Adam9* gave rise to all the Group II *tAdams*.

We found an intronless *Adam9b* containing a polyA tail in opossum, which represents one of the most recent retroposition events. Retroposed elements are found in high percentage in the opossum genome (Mikkelsen *et al.*, 2007). But we could not identify any other retroposed copy of other *Adams* in the opossum genome. The *tAdam* loci are prone to duplication and retroposition and hence provide excellent examples for studying gene duplication and evolution. In the future, it would be interesting to study the expression and function of the non-eutherian *tAdam* genes identified in this study.

FIGURES:

Figure 1: Structure of ADAM. The domain structure comprises of SP (signal peptide), PRO (prodomain), MP (metalloproteinase domain), DIS (disintegrin domain), CRD (cysteine-rich domain), EGF (epidermal growth factor-like domain), TM (transmembrane region), and CT (cytoplasmic tail).

Figure 2A: Bayesian tree of ADAMs for frog (*Xenopus tropicalis*).

Figure 2B: Bayesian tree of ADAMs for lizard (*Anolis carolinensis*).

Figure 2C: Bayesian tree of ADAMs for chicken (*Gallus gallus*)

Figure 2D: Bayesian tree of ADAMs for opossum (*Monodelphis domestica*).

Figure 2E: Bayesian tree of ADAMs for mouse (*Mus musculus*).

Figure 2F: Bayesian tree of ADAMs for human (*Homo sapiens*).

Figure 3: Syntenic analyses of the Group II *tAdams*. Most of the Group II *tAdams* lie next to *Adam9* in all vertebrates, except for *Adam2* in rodents.

Figure 4: Syntenic analysis of *Adam9-like* gene. Syntenic analysis of *Adam9-like* copy from lower non-mammalian vertebrates to cartilaginous elephant shark showed that it is highly conserved, while it disappeared in the course of evolution in higher mammals.

Figure 5: Comparison between *Adam9a* and *Adam9b* of opossum. (A) Location of *Adam9a* and *Adam9b* in the opossum genome. (B) Comparison table of *Adam9a* and *Adam9b* of opossum. (C) The gene structure of *Adam9b*. The *Adam9b* pseudogene contains a polyA sequence, and the only intron was identified as an opossum retroposon (Mdo3LTR1a/ERV1), which was likely inserted after the original retroposition event that generated *Adam9b*. The structure of the ADAM9a mRNA is shown. The black vertical lines in ADAM9a mRNA represent splice sites (Courtesy of Dr. Hari Bahudhanapati).

Figure 6: *Adam1* duplication. Expansion of *Adam1* through tandem duplication in chicken, opossum and mouse genome. *Adam1* gene is not found in the *X. tropicalis* genome and only one copy was found in the *Anolis* genome.

Table 1: Classification of mouse tADAMs.

Group	ADAMs
I	1, 4, 6, 20, 21, 24, 25, 26, 29, 30, and 34
II	2, 3, 5, 18 and 32

Note: This table is modified from Cho, 2012 by removing Group III consisting of ADAM7 and ADAM28.

Table 2. Accession numbers of ADAM proteins used in phylogenetic analyses.

Species	Protein Name	Accession Number
Human	ADAM2	Q99965
Human	ADAM7	Q9H2U9
Human	ADAM8	P78325
Human	ADAM9	Q13443
Human	ADAM10	O14672
Human	ADAM11	O75078
Human	ADAM12	O43184
Human	ADAM15	Q13444
Human	ADAM17	P78536
Human	ADAM18	Q9Y3Q7
Human	ADAM19	Q9H013
Human	ADAM20	O43506
Human	ADAM21	Q9UKJ8
Human	ADAM22	Q9P0K1
Human	ADAM23	O75077
Human	ADAM28	Q9UKQ2
Human	ADAM29	Q9UKF5
Human	ADAM30	Q9UKF2
Human	ADAM32	Q8TC27
Human	ADAM33	Q9BZ11
Mouse	ADAM1a	Q60813
Mouse	ADAM1b	Q8R534
Mouse	ADAM2	Q60718
Mouse	ADAM3	NP_033749
Mouse	ADAM4a	AAN77877
Mouse	ADAM4b	NP_001034084
Mouse	ADAM5	Q3TTE0
Mouse	ADAM6a	NP_777479
Mouse	ADAM6b	NP_001009545
Mouse	ADAM7	O35227
Mouse	ADAM8	Q05910.3
Mouse	ADAM9	NP_001257925
Mouse	ADAM10	O35598.2
Mouse	ADAM11	Q9R1V4.2
Mouse	ADAM12	Q61824.2

Mouse	ADAM15	NP_001257925
Mouse	ADAM17	Q9Z0F8.3
Mouse	ADAM18	Q9R157
Mouse	ADAM19	O35674.2
Mouse	ADAM21	Q9JI76
Mouse	ADAM22	Q9R1V6.2
Mouse	ADAM23	Q9R1V7.1
Mouse	ADAM28	Q9JLN6.3
Mouse	ADAM29	Q811Q4
Mouse	ADAM30	AAO38664
Mouse	ADAM32	Q8K410
Mouse	ADAM33	Q923W9.3
Mouse	TESTASE1/ADAM24	Q9R160
Mouse	TESTASE2/ADAM25	Q9R159
Mouse	TESTASE3a/ADAM26a	EQR158.2
Mouse	TESTASE3b/ADAM26b	NP_001009547
Mouse	TESTASE4/ADAM34	NP_665688
Mouse	TESTASE6/ADAM36	CAD65874
Mouse	TESTASE7/ADAM37	CAD65875
Mouse	TESTASE8 /ADAM38	AAI50794
Mouse	TESTASE9/ADAM39	NP_001020551
Opossum	ADAM1a	XP_001371130.2
Opossum	ADAM1b	XP_001379089.1
Opossum	ADAM2	XP_007476447.1
Opossum	ADAM4	XP_001376240.1
Opossum	ADAM7	XP_001372800.2
Opossum	ADAM8	XP_007486259.1
Opossum	ADAM9a	XP_001381888.1
Opossum	ADAM9b	XP_001371117.2
Opossum	ADAM10	XP_001377651.2
Opossum	ADAM11	XP_001375121.2
Opossum	ADAM12	XP_001367874.2
Opossum	ADAM15	XP_007482077
Opossum	ADAM17	XP_007476214.1
Opossum	ADAM18	XP_007476434
Opossum	ADAM19	XP_007474485
Opossum	ADAM20	XP_001376230
Opossum	ADAM22	XP_007505027
Opossum	ADAM23	XP_001370554
Opossum	ADAM28	XP_007477887
Opossum	ADAM30a	XP_007485363
Opossum	ADAM30b	XP_007485362

Opossum	ADAM30c	XP_001367215
Opossum	ADAM30d	XP_001376215.1
Opossum	ADAM32	XP_007476443
Opossum	ADAM33	XP_007496940
Chicken	ADAM8	XP_421552.4
Chicken	ADAM9	NP_001026567.2
Chicken	ADAM9L	XP_421805.4
Chicken	ADAM10	NP_989592.2
Chicken	ADAM11	XP_425842.4
Chicken	ADAM12	NP_001136322.1
Chicken	ADAM13	NP_001075887.1
Chicken	ADAM17	NP_001026567.2
Chicken	ADAM19	NP_001182051.1
Chicken	ADAM22	NP_001138700.1
Chicken	ADAM23	NP_001138702.1
Chicken	ADAM28	XP_004947615.1
Chicken	GrII tADAM1	XP_004947684.1
Chicken	GrII tADAM2	XP_003642600.2
Chicken	Grl tADAM1	NP_001264719.1
Chicken	Grl tADAM2	NP_996860.1
Chicken	Grl tADAM3	XP_003642244.2
<i>Anolis</i>	Grl tADAM1	XP_003228944.1
<i>Anolis</i>	Grl tADAM2	XP_008116367.1
<i>Anolis</i>	Grl tADAM3	XP_003225712.1
<i>Anolis</i>	Grl tADAM4	XP_008115539.1
<i>Anolis</i>	Grl tADAM5	XP_008116366.1
<i>Anolis</i>	Grl tADAM6	XP_008107167.1
<i>Anolis</i>	Grl tADAM7	XP_008116368.1
<i>Anolis</i>	GrII tADAM1	ENSACAG00000009283*
<i>Anolis</i>	GrII tADAM2	ENSACAG000000029425*
<i>Anolis</i>	ADAM9	XP_003226892.1
<i>Anolis</i>	ADAM9-L	XP_008104933.1
<i>Anolis</i>	ADAM10	XP_003228118.1
<i>Anolis</i>	ADAM11	XP_003222558.1
<i>Anolis</i>	ADAM12	XP_003218654.1
<i>Anolis</i>	ADAM13	XP_008120251.1
<i>Anolis</i>	ADAM17	XP_008115827.1
<i>Anolis</i>	ADAM19	XP_003223525.1
<i>Anolis</i>	ADAM22	XP_008110692.1
<i>Anolis</i>	ADAM23	XP_003223597.1
<i>Anolis</i>	ADAM28	XP_003226913.1

<i>Xenopus</i>	ADAM9	NP_001233130.1
<i>Xenopus</i>	ADAM9-L	XP_004915886.1
<i>Xenopus</i>	ADAM10	NP_001037869.1
<i>Xenopus</i>	ADAM11	NP_001243157.1
<i>Xenopus</i>	ADAM12	ABD52382.1
<i>Xenopus</i>	ADAM13	NP_001035102.1
<i>Xenopus</i>	ADAM15	ADK56765.1
<i>Xenopus</i>	ADAM16	AAI70249.1
<i>Xenopus</i>	ADAM17	NP_001182159.1
<i>Xenopus</i>	ADAM19	NP_001035105.1
<i>Xenopus</i>	ADAM22	O42596.2
<i>Xenopus</i>	ADAM23	NP_001233135.1
<i>Xenopus</i>	ADAM28a	NP_001120446.1
<i>Xenopus</i>	ADAM28b	NP_001233133.1
Platypus	ADAM9	XP_001519072.2
Zebrafish	ADAM9	NP_001004678.1
Zebrafish	ADAM9-L	NP_001107911.1
Medaka	ADAM9	XP_004072878.1
Medaka	ADAM9-L	XP_004080608.1
Shark	ADAM9	XP_007902115.1
Shark	ADAM9-L	XP_007883217.1

* Ensemble accession number

Table 3: Expression of *Adam9* and Group II *tAdams* transcripts in mouse and human.

Gene	Tissue	Mouse		Human	
		Transcript per million	Gene EST/Total EST in pool	Transcript per million	Gene EST/Total EST in pool
<i>Adam9</i>	adipose tissue	649	1/1540	77	1/12866
	adrenal gland	--	--	60	2/32940
	ascites	--	--	75	3/39834
	bladder	429	7/16283	133	4/29860
	bone	440	15/34066	--	--
	bone marrow	29	4/136333	--	--
	brain	33	16/475384	21	24/1092688
	connective tissue	504	10/19807	120	18/149072
	embryonic tissue	112	76/677554	56	12/212896
	epididymis	322	1/3101	--	--
	eye	80	15/185387	23	5/208840
	heart	36	2/54558	44	4/89524
	mammary gland	141	43/303048	--	--
	nerve	--	--	128	2/15535
	testis	32	4/121820	27	12/435204
	uterus	291	2/6855	21	5/232093
	sympathetic ganglion	400	4/9989	--	--
	vascular	--	--	271	14/51649
<i>Adam2</i>	brain	--	--	1	2/1092688
	connective tissue	--	--	20	3/149072
	lungs	10	1/99799	--	--
	prostate	--	--	10	2/189536
	testis	147	36/435204	82	36/435204
	thymus	2	1/121153	--	--
<i>Adam3</i>	brain	--	--	3	4/1092688
	spleen	86	8/92417	--	--
	testis	1264	154/121820	117	51/435204
<i>Adam5</i>	connective tissue	50	1/19807	46	7/149072
	eye	5	1/185387	--	--
	testis	582	71/121820	68	30/435204
<i>Adam18</i>	bladder	--	--	33	1/29860
	connective tissue	--	--	46	7/149072
	embryonic tissue	--	--	9	2/212896
	testis	82	10/121820	64	28/435204
<i>Adam32</i>	adipose tissue	--	--	77	1/12866
	brain	--	--	3	4/1092688
	connective tissue	--	--	114	17/149072
	embryonic tissue	7	5/677554	--	--
	kidney	--	--	4	1/210778
	testis	213	26/121820	124	54/435204

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Appendix

Figure 1: Bayesian tree of ADAM9 for all tested vertebrates.

Figure 2: Maximum likelihood tree for human ADAMs.

Figure 3: Maximum likelihood tree for mouse ADAMs.

Figure 4: Maximum likelihood tree for opossum ADAMs.

Figure 5: Maximum likelihood tree for lizard ADAMs.

Figure 6: Maximum likelihood tree for chicken ADAMs.

Figure 7: Maximum likelihood tree for frog ADAMs.

Figure 8: Maximum likelihood tree of ADAM9 for all tested vertebrates.

Figure 9: Neighbor joining tree for human ADAMs.

Figure 10: Neighbor joining tree for mouse ADAMs.

Figure 11: Neighbor joining tree for opossum ADAMs.

Figure 12: Neighbor joining tree for lizard ADAMs.

Figure 13: Neighbor joining tree for chicken ADAMs.

Figure 14: Neighbor joining tree for frog ADAMs.

Figure 15: Neighbor joining tree of ADAM9 for all tested vertebrates.