Characterizing the Expression and Function of FLRT2 in the ATDC5 Chondroprogenitor Cell Line

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science – Dentistry (Orthodontics)
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Abstract

Expression studies have implicated Fibronectin Leucine Rich Transmembrane protein 2 (FLRT2) in cranial neural crest cell migration and pre-chondrogenic cell condensation during craniofacial skeletogenesis. This aim of this study was to characterize the expression of FLRT2 and its relationship to the extracellular matrix (ECM) in ATDC5 chondroprogenitor cells.

Immunofluorescence studies localized FLRT2 to the cell membrane as well as extracellularly, where it colocalized with fibronectin. FLRT2 was identified in the ATDC5-derived ECM after cell extraction. Further to its colocalization with fibronectin, FLRT2 associated with fibronectin-coated beads in cell cultures. Co-immunoprecipitation confirmed that FLRT2 and fibronectin interact, either directly or indirectly. Blocking fibronectin fibril formation in ATDC5 cell cultures demonstrated a concomitant decrease in extracellular FLRT2 accumulation. It appears that FLRT2 may exist in both a membrane-bound and a shed form. Either or both of these forms may participate in cell-ECM interactions in cooperation with fibronectin or other ECM proteins.
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List of Abbreviations

Cranial Neural Crest Cells = CNCC
Ectodomain = ECD
Extracellular Matrix = ECM
Fibronectin Leucine Rich Transmembrane protein = FLRT
Fibronectin Type III domain = FNIII
Glycosaminoglycan = GAG
Leucine Rich Repeats = LRR
Neural Cell Adhesion Molecule = NCAM
Poly-L-lysine = PLL
Original Contributions and Collaborations

I performed all of the work presented in this thesis under the supervision of Dr. Siew-Ging Gong and my advisory committee (Dr. Morris Frank Manolson and Dr. Christopher McCulloch). Dr. Wilson Lee provided the polycarboxylate-coated microbeads and protocols for coating beads. Dr. Yamini Arudchelvan performed the immunoprecipitation and immunoblotting experiments related to Figure 3-11.
Chapter 1
Introduction and Background

1 Introduction

Craniofacial development is a complex process involving the fine-tuned temporal and spatial coordination of several important processes. Whereas many of these processes have been well described on a macroscopic level, rapid advances in molecular and cell biology techniques are providing the means to gain a greater insight into the underlying cellular pathways. A striking example of this is the discovery of single genes implicated in craniofacial disorders such as craniosynostoses (FGFR), mandibulofacial dysostosis (TCOF-1), and cleidocranial dysplasia (CBFA1). Analysis of the roles of these genes reveals that an alteration in a single receptor or transcription factor can have wide-reaching ramifications. A critical process in craniofacial development is the condensation of groups of cells that subsequently undergo differentiation and ultimately form the structures of the face. It has previously been shown that a member of the Fibronectin Leucine Rich Transmembrane (FLRT) gene family, FLRT2, is highly expressed in an area of cell condensation that eventually undergoes chondrogenic differentiation. The focus of my study is to characterize the potential role of a protein, FLRT2, in mediating cell-ECM interactions that may be important during pre-chondrogenic cellular condensation. A review of the literature pertaining to some of the early cellular events during craniofacial development will be presented, including cellular condensation and the way in which cells interact with the surrounding environment during the condensation process. The composition/function of and cellular interactions with the ECM will be discussed in brief. Fibronectin, being implicated in the present experimental work, will be discussed in more detail. Finally, the experimental methods, findings, and a discussion of their implications for the role of FLRT2 will be presented.
1.1 Craniofacial Skeletogenesis – A Broad Perspective

By about the third week of embryonic development, the three primary germ layers (ectoderm, mesoderm, and endoderm) are formed. Neurulation is initiated and results from the thickening of a portion of embryonic ectoderm, the neural plate, which begins to invaginate along its central axis with both sides folding over to eventually fuse and form a tube. As these neural folds are fusing, a specialized population of neuroectodermal cells begins to lose attachment from the neighbouring epithelium. These cells, referred to as the neural crest cells, begin to migrate ventrally along the sides of the neural tube towards their final destinations. The neural crest cells eventually become widely distributed throughout the body to give rise to various ganglia, nerve sheaths (Schwann cells), the meningeal covering of the brain, pigment cells, and the adrenal medulla (Moore, Persaud 1993, Sperber, Sperber et al. 2010). In the most rostral portion of the developing neural tube, the population of neural crest cells is called cranial neural crest cells (CNCC). A portion of the CNCC migrate to the developing pharyngeal arches and frontonasal area early in the fourth week of development (Gilbert 2000). Upon arriving at their final destinations, the CNCC (now referred to as the “ectomesenchyme”) interact with the surrounding epithelium (Santagati, Rijli 2003). These epithelial-mesenchymal interactions are important in initiating cellular condensation, marking the beginning of cellular differentiation that precedes the formation of many of the cartilaginous and skeletal structures of the head and neck (Hall, Miyake 1992). In the developing frontonasal region, for instance, the CNCC that rest in the midline of the merged medial nasal prominences undergo condensation and differentiate into chondrocytes that go on to form the cartilaginous nasal septum. Similar events occur throughout the frontonasal process and first two branchial arches, with the cranial neural crest cells giving
rise to most of the skeletal elements of the face (Moore, Persaud 1993, Sperber, Sperber et al. 2010).

1.2 Cell Condensation

Cellular condensation is crucial to the development of many of the body’s structures. It was first described by Dame Honor Fell in 1925 (Fell 1925). Grüneburg later referred to cellular condensation as “the membranous skeleton” to describe it as a distinct and fundamental stage in skeletal development (Grüneberg 1963). In relation to craniofacial skeletogenesis, condensation of the ectomesenchyme results in the terminal differentiation of these cells which then begin to secrete structure specific matrix such as cartilage, bone, enamel, and dentin (Hall, Miyake 2000).

Cellular condensations are characterized by a localized increase in cell density and are a result of a combination of either: 1) increased mitotic activity, 2) aggregation of cells toward a centre, or 3) reduced movement of cells away from a centre (Hall, Miyake 1995). The mechanisms used to induce cellular condensation appear to differ depending on the location within the developing organism. For example, increased mitotic activity does not appear to play a part in forming condensations in the developing limb buds. Conversely, it is believed that proliferative signals generated from epithelial-mesenchymal interactions are an important factor in mediating cellular condensations in the craniofacial complex (Thorogood, Hinchliffe 1975, Epperlein, Lehmann 1975, Hall, Miyake 1992). A critical aspect in all cases of cell condensation is the need for cells to interact with both adjacent cells and with the assembly of proteins that surrounds them - the ECM.

1.3 The Extracellular Matrix

All cells make contact with the ECM, either continuously or transiently during important phases in their lives. The ECM has traditionally been thought of as merely a structural support for the
surrounding cells. More recent research indicates that, in addition to acting as a scaffold for cell adhesion and migration, the ECM participates in cell signaling, provides mechanical characteristics that can change cell behaviour, and acts as a reservoir for both soluble and solid state growth factors (Discher, Mooney et al. 2009, Geiger, Spatz et al. 2009, Berrier, Yamada 2007, Hynes 2009).

Proteins of the ECM tend to be highly conserved, indicating that their complex sequences are of biological and evolutionary importance. The major classes of ECM molecules include adhesive glycoproteins (fibronectin, vitronectin, and laminin), collagens, elastin and microfibrillar proteins, matricellular proteins, and proteoglycans (Mecham 2001):

Adhesive glycoproteins such as fibronectin and vitronectin interact directly with cell surface proteins. This usually occurs through structural components that are only present on fully folded proteins or that may be cryptic (i.e. hidden) in nature. One well-characterized example is the Arg-Gly-Asp (RGD) sequence (present on both fibronectin and vitronectin) that is recognized by cell surface integrins (Ruoslahti 1996). Fibronectin is a large glycoprotein that is found in a soluble form (plasma fibronectin) as an insoluble, ECM-bound form (cellular fibronectin). The soluble form is fairly inactive, while the insoluble form is involved in a variety of cellular interactions with the ECM. Fibronectin’s diverse functions include roles in cell adhesion, migration, growth, and differentiation (Pankov, Yamada 2002). Cellular fibronectin has been shown to be crucial to life: mouse embryos lacking fibronectin expression have major lethal defects (George, Georges-Labouesse et al. 1993). These fibronectin-null mouse embryos displayed neural tube abnormalities and severe defects in mesodermally derived tissues. Experiments in Xenopus embryos have shown that fibronectin-mediated cell protrusions are essential for mesodermal migration, while migrating avian neural crest cells were found to
express a variety of fibronectin receptors (Winklbauer, Keller 1996, Testaz, Delannet et al. 1999). Fibronectin is secreted as a dimer of approximately 250kDa, with each monomer unit consisting mainly of three types of repeat sequences. The type I (FN1) repeats are 40 amino acids in length and contain two disulfide bonds. The type II (FNII) repeats are 60 amino acids and also contain two disulfide bonds. Each fibronectin monomer contains twelve type I and two type II repeats. The type III (FNIII) repeats are 90 amino acids long, do not contain any disulfide bonds, and vary in number from fifteen to seventeen. Cell-type-specific alternative splicing can generate as many as 20 different isoforms of fibronectin in humans (Pankov, Yamada 2002, Singh, Carraher et al. 2010, Mecham 2001).

Fibronectin binding to cells is mediated mainly by cell-surface integrins, with the most well-characterized example being the binding of α5β1 integrin to the RGD sequence in the 10th FNIII repeat (Singh, Carraher et al. 2010, Pankov, Yamada 2002). In addition to interacting with integrins, fibronectin has binding sites for various molecules and bacteria, including collagen, gelatin, chondroitin sulfate proteoglycan, heparin, fibrin, *Staphylococcus aureus*, and importantly, itself (Mecham 2001). Extracellular assembly of fibronectin fibrils is a cell-mediated process whereby fibronectin dimers bind to cell surface integrins, initiating receptor clustering and fibronectin-fibronectin interactions that are mediated by the N-terminal ends of the protein (Singh, Carraher et al. 2010). Further conformational changes expose cryptic sites that allow for more fibronectin interactions and subsequent fibril assembly (Hocking, Sottile et al. 1994). Thin fibrils dominate early on, which are then assembled into thicker bundles (Singh, Carraher et al. 2010).

*Collagens* are the most ubiquitous ECM proteins and are designed to provide structural support to tissues. Nineteen different types of collagen have been described and are differentially
distributed throughout the tissues of the body. Cartilage, for instance, contains mainly type II collagen, forming a network of fibrils that supports the matrix (Eyre 2002).

Elastin, unlike collagen, is encoded by a single gene. It is particularly important in tissues where air or blood flow require constant changes in volume (i.e. lungs, blood vessels). When the central portion of elastin is stretched, the hydrophobic core of the protein is exposed. Subsequent shielding of the exposed hydrophobic amino acids causes the protein to collapse back upon itself, giving elastin the important property of recoil (Kielty, Sherratt et al. 2002).

Microfibrils (e.g. fibrillin-1 and fibrillin-2) are abundant in elastic tissues, though they are found throughout the body. Their association with developing elastic fibers has led to the hypothesis that they are involved in elastin assembly (Mecham 2001).

Matricellular proteins do not contribute to the structural integrity of the ECM but instead provide a link between the cell and the ECM by binding to both ECM proteins and cell surface receptors. Tenascin-C, for example, is transiently expressed in the ECM along the migration pathways of CNCC and at the boundaries of pre-chondrogenic cell condensations (Koyama, Shimazu et al. 1996, Mackie, Tucker et al. 1988). Interestingly, tenascin-C contains cell-binding sites but has been shown to have inhibitory effects on cell attachment to other ECM proteins such as fibronectin and laminin (Mecham 2001).

The proteoglycans are a large group of over 25 proteins. These consist of core proteins with attached glycosaminoglycan (GAG) chains. They are structurally and functionally diverse but can be classified into 4 broad categories: 1) large proteoglycans that form aggregates by interaction with hyaluronan, 2) basement membrane proteoglycans, 3) cells surface heparan sulfate proteoglycans, and 4) small leucine-rich proteoglycans (Mecham 2001). Aggrecan is a member of the first category of proteoglycans and is an important constituent of cartilage. The
high density of attached GAG chains produces an osmotic pressure that allows for absorption and redistribution of water, giving cartilage its compressive strength (Watanabe, Yamada et al. 1998). Syndecan, which is known to regulate cell condensation boundaries by binding to fibronectin, is an example of a cell surface heparan sulfate proteoglycan (Hall, Miyake 2000). Proteins from the last group, the small leucine-rich proteoglycans, possess leucine rich repeat (LRR) motifs. The arc shape that this sequence assumes allows these proteins to interact with numerous ECM molecules as well as with growth factors (Mecham 2001, Enkhbayar, Kamiya et al. 2004, Kobe, Kajava 2001).

Most of the proteins in the ECM are quite large with complex sequences and many exist as alternatively spliced variants that differ in function (Mecham 2001, Hynes 2009). During chondrogenesis, for example, a splice variant of fibronectin containing an extra type III domain (IIIA) is specifically upregulated and shown to be essential for the cellular condensation phase (Gehris, Stringa et al. 1997). A different splice variant of fibronectin containing an alternate extra type III domain (IIIB) is also expressed during cartilage formation and is maintained at high levels throughout chondrogenesis (Gehris, Oberlender et al. 1996). The existence of two forms of the same protein that show tightly regulated spatial and temporal expression highlights the importance of a single domain in altering an ECM protein’s functionality.

Conventional cell-ECM interactions involve cell surface receptors that recognize portions of the ECM and convey signals to the interior of the cells (Hynes 2002, Berrier, Yamada 2007). The ECM may further contribute to cell signaling via several possible mechanisms: 1) the retention of soluble growth factors, 2) the generation of soluble growth factors via degradation of solid state proteins, 3) ECM proteins intrinsically acting as growth factors in the solid state, and 4) ECM proteins interacting with ligands as “cofactors” to orchestrate binding between
extracellular proteins and cell-surface receptors (Hynes 2009). An example of the interplay between the ECM and growth factors is the specific expression of the type IIa procollagen isoform in prechondrogenic tissues. This form of procollagen contains an extra sequence that, unlike other forms of the pro-peptide sequence, remains attached to collagen when it is deposited into the ECM. This sequence has been shown to bind TGF-β and BMP-2 and thus regulate signaling during early cartilage formation (Zhu, Oganesian et al. 1999).

1.3.1 Cell-ECM Interactions

It is becoming increasingly apparent that the ECM plays a very dynamic role in many of the cellular processes that are important during development, and that areas of contact between cells and the ECM are crucial to these processes. Cell surface receptors can interact with ECM molecules that in turn can trigger signals inside the cell. Cells can also adhere to the ECM at specialized cell surface structures, termed “matrix adhesions”, that are intimately connected to the ECM (Berrier, Yamada 2007).

Prior to around 2001, cell adhesions had been divided into two distinct types – focal adhesions and fibrillar adhesions. Much of the information generated on these structures was derived from experiments using cells grown on planar substrates, and it later became apparent that cells grown in 3D substrates demonstrated adhesions that were distinct from classical fibrillar and focal adhesions. These “3D adhesions” were associated with altered cellular morphology and migration properties (Cukierman, Pankov et al. 2001).

At least 4 types of cell-ECM adhesions have been identified in fibroblasts (Cukierman, Pankov et al. 2001, Cukierman, Pankov et al. 2002, Berrier, Yamada 2007):

1) Focal complexes: Early, transient cell attachments to the ECM.
2) Focal adhesions: Stabilized cell-ECM attachments (derived from focal complexes). Characterized by $\alpha_v\beta_3$ integrin (a receptor for vitronectin), vinculin, and tyrosine phosphatase proteins.

3) Fibrillar adhesions: Derived from focal adhesions and characterized by $\alpha_5\beta_1$ integrin (a receptor for fibronectin) and tensin.

4) 3D matrix adhesion: Occur in cells grown on 3D substrates. Characterized by a predominance of $\alpha_5\beta_1$ integrin (with some $\alpha_v\beta_3$ integrin), vinculin, paxillin, and actinin.

Integrins are the main receptors responsible for mediating cell-ECM interactions. They are a group of heterodimeric receptors composed of pairings between 18 alpha and 8 beta subunits. Twenty-four such receptors have been described - each of these is expressed in different tissues and the extracellular portion binds to different components of the ECM, cell surface proteins, matricellular proteins, and matrix proteases (Miranti, Brugge 2002, Luo, Carman et al. 2007). Their intracellular portions form complexes with cytoplasmic proteins that connect to the cytoskeleton and downstream signaling pathways (Hynes 2002). Binding to ligands triggers integrin receptor clustering and activates cytoplasmic receptors such as focal adhesion kinase, serine/threonine kinases, lipid kinases, and phosphatases (Li, Guan et al. 2005, Lo 2006). The downstream effects can alter the cytoskeleton, gene regulation, and other cellular processes. Integrins convey information in a bidirectional manner – thus, the ECM can influence the inside of the cell and vice versa.

### 1.4 Cell Interactions During Condensation

#### 1.4.1 Cell-Cell Interactions in Condensations

During pre-chondrogenic cellular condensation, specific cell surface molecules are upregulated, allowing for an increase in cell-cell contacts. Gap junctions form between adjacent cells and
allow for the transfer of small molecules between them (Zimmermann 1984). In the developing frontonasal region, for instance, an increase in connexins (the proteins associated with gap junctions) has been shown to precede chondrogenesis of the nasal septum (Loty, Foll et al. 2000). N-cadherin, a calcium-dependent cell adhesion molecule, and neural cell adhesion molecule (NCAM) are both increased in cellular condensations (Chuong, Edelman 1985, Edelman 1986, Hall, Miyake 1995, Tavella, Raffo et al. 1994, Widelitz, Jiang et al. 1993). N-cadherin is believed to be responsible for mediating cellular interactions that initiate condensation (Oberlender, Tuan 1994) whereas NCAM, although not required for the onset of cellular condensation, plays a role in setting their boundaries.

1.4.2 Cell-ECM Interactions in Condensations

The ECM provides signals that push cells towards differentiation. For example, mesenchymal cells removed from embryonic chick wing bud condensations and cultured on collagen gels lost their condensation-specific cell-surface phenotype. Despite being removed from their normal condensation environment, these cells were able to synthesize collagen II, indicating that the presence of extracellular collagen supported chondrogenic differentiation (Aulthouse, Solursh 1987). Indeed, several ECM molecules have been shown to be highly expressed in cell condensations and are important to the process.

Fibronectin is upregulated during cellular condensation (Kulyk, Upholt et al. 1989, Ohya, Wantanabe 1994). Fibronectin is induced by TGF-β and in turn it activates NCAM (Hall and Miyake, 2000). Temporal expression patterns of N-cadherin and fibronectin suggest that condensations proceed from an N-cadherin dependent stage through to a fibronectin-dependent stage (Kawai, Akiyama et al. 1999). Though it is an ECM protein, fibronectin is intimately related to cell surface receptors during and after assembly, and it is believed to contribute
directly to cell-cell interactions in addition to its indirect mechanism via upregulation of NCAM (Frenz, Jaikaria et al. 1989). A specific splice variant of fibronectin (containing exon IIIA) is found in high levels during condensation and is downregulated thereafter (Gehris, Oberlender et al. 1996). Antibodies against the exon IIIA region of fibronectin have been shown to ablate cellular condensation and inhibit limb development both in vitro and in vivo, demonstrating the necessity for this fibronectin isoform during normal development (Gehris, Stringa et al. 1997).

Several other ECM proteins have been localized to cellular condensations. Syndecan, a cell surface proteoglycan, sets the temporal and spatial boundaries of cellular condensations through two mechanisms: 1) binding to tenascin, a “matricellular protein”, and 2) binding to and inactivating fibronectin with the downstream effect of NCAM inactivation (Thesleff, Vainio et al. 1990, Salmivirta, Elenius et al. 1991, Shames, Jennings et al. 1991). In addition to the ECM proteins listed above, cellular condensations are characterized by the presence of elevated levels of versican, hyaladherins, heparan sulphate proteoglycans, and chondroitin sulphate proteoglycans, though less is known about their respective roles (Hall, Miyake 1995, Hall, Miyake 2000).

1.4.3 Mutations Affecting Cellular Condensation

Various disorders in skeletal development have been shown to be due to mutations that exert their effects at the cellular condensation stage, highlighting the importance of cell-cell and cell-ECM interactions in forming condensations of the appropriate size and at the appropriate time.

Brachypod mutant mice display abnormal pre-chondrogenic condensations (Gruneberg, Lee 1973). These mice contain a mutation in Growth-Differentiation Factor 5 (GDF-5) a member of the TGF-β superfamily. This mutation results in altered adhesive properties of cells during
condensations, manifesting phenotypically as agenesis of distal limb elements and associated proximal limb element hypotrophy (Storm, Huynh et al. 1994, Hall, Miyake 1995).

Talpid\textsuperscript{3} is a mutation found in domestic fowl and is typified by cartilaginous elements that are either fused or duplicated (Ede, Kelly 1964, Ede, Hinchliffe et al. 1971, Johnson 1986). Cells in condensations are overly adhesive and less motile due to elevated levels of NCAM, resulting in abnormally large pre-chondrogenic cellular condensations that ultimately translate into inappropriate cartilage formation (Hall, Miyake 1995).

The importance of regulating condensation size within an appropriate range is well demonstrated by the lethal congenital hydrocephalous (\textit{ch}) mutation in mice. Both pre-chondrogenic and pre-osteogenic condensations are affected, leading to widespread skeletal defects. Pre-chondrogenic condensations are too small, causing agenesis and abnormal development of most of the cartilaginous skeleton. Conversely, pre-osteogenic condensations are too\textit{ large}, leading to early and excessive formation of membranous bone (Grüneberg 1963, Gruneberg, Wickramaratne 1974, Hall, Miyake 1992).

In line with Grüneberg’s intial observations, the above mutations provide striking evidence in support of the importance of cellular condensation in skeletal development. It is clear that the tight coupling of cellular proliferation, cell-cell interactions, and cell-ECM interactions is required to orchestrate this process in the developing craniofacial complex.

### 1.5 The FLRT Gene Family

The FLRT protein family was first described by Lacy \textit{et al} (1999) after a screen designed to isolate ECM proteins expressed in muscle identified \textit{FLRT1}. They then went on to identify the other two members of the \textit{FLRT} family in a sequence library. The FLRT proteins are type I
transmembrane proteins, characterized by an extracellular domain containing 10 LRRs flanked by N- and C-terminal cysteine rich regions and a FNIII domain (figure 1.1). Their relatively short intracellular tails lack any sequence homology (Lacy, Bonnemann et al. 1999). Although the three members of the FLRT protein family share a common structural theme, their spatial and temporal expression differs during development and into adulthood, indicating that they have diverse functions (Lacy, Bonnemann et al. 1999, Haines, Wheldon et al. 2006, Gong, Mai et al. 2009).

Figure 1-1: Diagrammatical representation of the common structural features of FLRT proteins. The extracellular domain contains 10 LRR sequences flanked by N- and C-terminal cysteine-rich regions. The FNIII domain lies closer to the cell membrane. These proteins span the cell membrane once and have a short cytoplasmic tail.

The LRR motifs that comprise about 50% of the FLRT’s structures may provide some insight into the function of these proteins. LRR motifs were first recognized and characterized in a protein isolated from human serum but have been identified in over 2000 proteins from viruses, plants, arcaea, and eukaryotes (Takahashi, Takahashi et al. 1985, Enkhbayar, Kamiya et al. 2004). These proteins encompass a diverse range of functional classes such as tyrosine kinase
receptors, cell adhesion molecules, bacterial virulence factors, enzymes, and ECM-binding glycoproteins (Enkhbayar, Kamiya et al. 2004). While this list of roles appears to be quite divergent, it is important to note that all of these functions ultimately encompass a broader role in mediating protein-protein interactions. The LRR motifs are thought to form a concave surface that is ideal for protein-protein interactions (Kobe, Deisenhofer 1994, Kobe, Kajava 2001). Indeed, several known LRR-containing proteins have been shown to interact with ligands via the concave surface formed by these LRR sequences (Enkhbayar, Kamiya et al. 2004).

Other structural features of the FLRT proteins may provide further clues about their functions. The cysteine-rich sequences that flank the LRR sequences are found in many other LRR proteins and are proposed to contribute to the tertiary structure of the protein or to be involved in forming disulfide bonds that stabilize protein-protein interactions (Kobe, Kajava 2001). The FNIII domain is found in cell surface receptors and ECM proteins such as fibronectin, tenascin, NCAM, and receptor protein tyrosine phosphatase-β (Leahy, Hendrickson et al. 1992, Kasper, Stahlhut et al. 1996, Adamsky, Schilling et al. 2001). These FNIII domains are often involved in coordinating interactions between cell surface receptors and ECM molecules, thus implying that FLRT proteins may be involved in adhesive events.

1.6 FLRT2

Of the three family members, FLRT2 is the most well-characterized in terms of craniofacial development. It is expressed along migration paths of CNCC towards the developing pharyngeal arches. FLRT2 is also expressed in the vomeronasal organ, the developing frontonasal mass, at sites of epithelial-mesenchymal interactions (i.e. the developing tooth bud, hair follicle, and eye) and in the posterior palatal shelves before, during, and after fusion (Gong, Mai et al. 2009). Further along in craniofacial development, FLRT2 is highly expressed in the
region of the developing frontonasal process that ultimately differentiates to form the
cartilaginous nasal septum. The expression of *FLRT2* in the frontonasal region is correlated with
genes that are known to be important players in craniofacial development, specifically in
patterning and chondrogenesis. The expression of *Msx1*, a gene that has been described as a
repressor of chondrogenic differentiation, was mutually exclusive of that of *FLRT2* in this area.
Conversely, *FLRT2* and *Fgfr2* expression were overlapping, correlating with *in vitro* data
showing that these two proteins physically interact (discussed below) and providing further
evidence that FLRT2 may be involved in regulating FGF signaling. Finally, *FLRT2* expression
was shown to underlie an area with expression of *Shh*, a gene that has been shown to promote
chondrogenesis in the developing frontonasal region in cooperation with *FGF8* (Gong, Mai et al.
2009, Bottcher, Pollet et al. 2004). These correlations, along with the prominent expression of
*FLRT-2* in the developing frontonasal region provide additive evidence indicating a potential role
in chondrogenesis in this area.

In addition to its expression in the developing craniofacial region, *FLRT2* was also shown to be
expressed in human adult pancreatic, skeletal muscle, brain, and heart tissues (Lacy, Bonnemann
et al. 1999). Conversely, the pattern of *FLRT2* expression throughout embryological
development is distinct from that seen in adult tissues and suggests that FLRT2 has a crucial role
in certain embryological processes. Haines *et al* (2006) demonstrated *FLRT2* expression in the
sclerotome, cephalic mesoderm, branchial arches, and the epithelia of the body wall of
developing mouse embryos.
1.6.1 Molecular Weight, Cellular Expression, and Localization of FLRT2

The expression of FLRT2 on a cellular level has been investigated using both transfected and natively expressing cell lines. Interestingly, these studies have yielded variable data with respect to molecular weight. The predicted molecular mass of FLRT2 is around 75 kDa; there are, however, 5 potential N-glycosylation sites (Lacy, Bonnemann et al. 1999). Immunoprecipitation of FLRT2 transfected COS-1 cells with an anti-FLRT2 peptide antibody (figure 1-2, a) yielded a protein that migrated at 85kDa, which, upon treatment with a deglycosylating enzyme, resulted in a band running at a lower molecular weight in western blots (Lacy, Bonnemann et al. 1999). A later study of FLRT2 expression in the similar COS-7 cell line (figure 1-2, b) corroborated the fact that FLRT2 was glycosylated; however, the resulting molecular weights of glycosyated (100kDa) and unglycosylated (85kDa) FLRT2 differed from the previous study (Haines, Wheldon et al. 2006). Western blot analysis of FLRT2 expression in transfected HEK293T cells and primary mouse neurons that natively express the protein (figure 1-2, c) revealed a band running at 100kDa in both cell lines tested, corroborating the data generated by Haines et al. (2006) (Yamagish, Hampel et al. 2011). More recently, Gong et al. (2009) characterized endogenous FLRT2 expression in the ATDC5 chondroprogenitor cell line. Interestingly, while the primary antibody used to detect FLRT2 in the ATDC5 cell lysates was the similar to that employed by Yamagish et al (2011), the resulting FLRT2 band had a much lower molecular weight of around 75kDa as compared to the 100kDa band generated in their experiments.
Figure 1-2 (a-c): Expression of FLRT2 in various cell lines. (a) $^{35}$S-labeled SF9 and COS-1 cells expressing FLRT2 constructs were lysed and immunoprecipitated with a FLRT2-specific antibody, followed by incubation with (+) or without (-) a deglycosylating enzyme. Bands representing glycosylated FLRT2 were present at around 85kDa in both cell lines. Deglycosylation resulted in bands running at a lower molecular weight. (b) Western blot of FLRT2-transfected COS-7 cell lysates detected with an antibody recognizing a 3’ HA tag present on the intracellular portion of the protein. Cells were cultured either with (+) or without (-) tunicamycin to prevent glycosylation. Glycosylated FLRT2 is present at around 100kDa. Lower molecular weight bands running at around 20kDa are present in both samples, likely representing the juxtamembrane and intracellular portions of FLRT2 remaining after cleavage of the ECD. (c) E16.5 mouse cortical neurons were cultured in vitro for 6 days. Total cell lysates (TCL) and cell-conditioned media (Media) were immunoblotted with a monoclonal antibody recognizing an extracellular epitope on FLRT2 ($\alpha$-FLRT2-ECD). Cell lysates demonstrated the full-length FLRT2 protein running at around 100kDa. Cell-conditioned media yielded two bands running at lower molecular weights, representing shed FLRT2 ECD. Reprinted by permission from Lacy et al. (a), Haines et al. (b), and Yamagishi et al. (c), with permission from Elsevier and Nature Publishing Group.

It is unclear whether the disparities described above are due simply to inter-experimental variability or whether they can be attributed to cell-dependent differences in post-translational processing of FLRT2. It is interesting to consider whether there might actually be different forms of FLRT2 that can act in different ways depending on their environment. Interestingly, some evidence suggests that FLRT2 is found in a soluble form. Whereas immunofluorescence
images of transfected COS-1 and HEK293T cells suggest that FLRT2 is localized to the cell membrane, other findings point to the possibility of an extracellular form (Haines, Wheldon et al. 2006, Karaulanov, Bottcher et al. 2006). Haines et al (2006) described an additional 20kDa band running on FLRT2 western blots using transfected COS-7 cell lysates (figure 1-2, b). Based on molecular weight calculations, they believed that this corresponded to the juxtamembrane and intracellular portion of the protein, indicating that the extracellular portion of FLRT2 had been cleaved. More recently, Yamagishi et al (2011) provided striking evidence demonstrating shed FLRT2 ectodomains (ECD) in cell-conditioned media from both primary mouse neurons (figure 1-2, c) and FLRT2-transfected HEK293T cells (figure 1-3). Cell-conditioned media yielded two bands migrating at 75kDa and 85kDa, indicating two potential cleavage sites. These were mapped to sequences flanking a conserved N-glycosylation sequence in the juxtamembrane region of the protein (figure 1-3). Thus, it appears likely that FLRT2 is found in a shed, soluble form as well as a membrane-bound form. Whether there is a difference in function between these two forms of the protein remains to be seen.
1.6.2 FLRT2 in FGF Signaling

The FGF signaling pathway is highly important in development, particularly in the formation of the craniofacial skeleton (Nie, Luukko et al. 2006). In light of this, it is interesting to consider the multitude of evidence supporting a relationship between FLRT2 and FGF signaling. In *Xenopus* embryos, FLRT2 gain and loss of function mutations phenocopied FGF signaling (Bottcher, Pollet et al. 2004). More specifically, anti-FLRT2 morpholino oligonucleotides blocked the FGF8 mRNA-dependent induction of neural markers in *Xenopus* animal caps, indicating that FGF signaling may be dependent on FLRT2. Haines *et al* (2006) demonstrated that FLRT2 interacts with FGFR1 independent of receptor activation. Similarly, treatment of HEK293T kidney cells with FGF-2 resulted in an up-regulation of FLRT2 gene expression.
FLRT2 has also been shown to interact directly with FGFR2 in tissue lysates derived from the craniofacial region of embryonic mice. The interaction between FLRT2 and FGFR2 was shown to be a direct one, with the FLRT2 LRR sequence contributing to the interaction with the extracellular portion of FGFR2 and the FLRT2 C-terminal sequence contributing to the interaction with the intracellular portion of FGFR2. Increasing and decreasing FLRT2 expression in the ATDC5 chondroprogenitor cell line caused a concomitant increase and decrease in FGFR2 expression, suggesting a positive feedback relationship (Wei, Xu et al. 2011).

1.6.3 FLRT2 in Cell Adhesion

Because FLRT2 contains a fibronectin-like portion in its extracellular domain, Lacy et al (1999) initially hypothesized that it might be involved in adhesion. Further on, studies provided evidence in support of this hypothesis. A series of experiments using HEK293T cells transfected with FLRT2-GFP constructs demonstrated that FLRT2 expression led to homotypic cell sorting in a calcium-dependent manner, similar to cadherin-transfected cells (Karaulanov, Bottcher et al. 2006). FLRT2 has also been shown to co-localize with vinculin in transfected COS-7 cells, suggesting that it is involved in mediating cell-cell adhesion. When cells were not in contact, FLRT2 was present around the entire cell membrane, whereas it became concentrated at projections connecting cells once they were in contact (Haines, Wheldon et al. 2006). Whether this cell-sorting or adhesive behaviour can be attributed to an ability of FLRT2 to interact directly with itself remains a controversial issue. Co-immunoprecipitation and bioluminescence resonance energy transfer (BRET) assays with FLRT2-transfected cells provided evidence suggesting that FLRT2 participates in homotypic interactions (Karaulanov, Bottcher et al. 2006). Conversely, later experiments by different investigators using the same HEK293T cell line indicated that FLRT2 does not interact with itself (Yamagishi, Hampel et al. 2011).
1.6.4 FLRT2 in Migration

The expression of FLRT2 along CNCC migration pathways indicates that it may also have some involvement in cell movement. Two studies, using very different methodology, have provided evidence for this. The ECD of FLRT2 was shown to be shed from the surface of primary mouse neurons, with the ECD acting as a soluble chemorepellent to migrating Unc5D-positive neurons (Yamagishi, Hampel et al. 2011). In another study, over-expression of FLRT2 in the ATDC5 chondroprogenitor cell line resulted in increased cell migration, while knockdown of FLRT2 inhibited cell migration in a wound assay (Xu, Wei et al. 2011). This raises the very interesting possibility that FLRT2 contributes to cell-ECM interactions and may do so in either a cell-bound or a soluble form (or perhaps both). The function and form of FLRT2 may vary in a cell- and environment-dependent manner, which might help to explain why different researchers have identified FLRT2 at different molecular weights depending on the cell line in which it was expressed.

1.6.5 FLRT2 in Cellular Condensation and Chondrogenesis

The marked expression of FLRT2 in the developing frontonasal region is mutually exclusive of MSX1, a repressor of chondrogenesis, while it overlaps with the cartilage-promoting FGF and SHH. This led Xu et al. (2011) to examine the role of FLRT2 during cell condensation and chondrogenesis. Stable FLRT2 overexpressing and knockdown cell lines were created using the ATDC5 chondroprogenitor cell line and it was found that FLRT2 expression affected various cellular characteristics. FLRT2 expression was directly correlated to the rate of cellular proliferation during the first 14 days of culture, suggesting that it is involved in the regulation of cell division. FLRT2 expression was inversely related to the level of N-cadherin expression as well as the number of PNA-positive prechondrogenic cell condensations, indicating that it may play a role in regulating the level of cell-cell adhesion. Despite there being fewer visible cell
condensations in the FLRT2 overexpressing cells, these cultures ultimately produced more Alcian blue-positive cartilage matrix after 14 days of culture, indicating a potential increase in chondrogenic differentiation. It is interesting to note that this is contrary to what would be anticipated, as there is normally a direct relationship between condensation number/size and the subsequent degree of chondrogenic differentiation (Hall, Miyake 2000). This body of evidence clearly implicates FLRT2 in cellular condensation and chondrogenesis. Its exact role here, however, remains unclear and is an area that is definitely worthy of further investigation.

1.7 FLRT3

The structure of FLRT3 is closely related to that of FLRT2. There is some overlapping expression of these 2 genes during development as well as evidence that they are functionally interchangeable during heart morphogenesis (Lacy, Bonnemann et al. 1999, Gong, Mai et al. 2009, Muller, Schulz et al. 2011). Similarly, FLRT2 and FLRT3 promote the same sort of homotypic cell sorting behaviour in transfected HEK293T cells (Karaulanov, Bottcher et al. 2006). Because the functions of FLRT2 and FLRT3 may be similar, a brief consideration of the body of evidence looking specifically at the function of FLRT3 may provide some useful insight.

FLRT3 has been shown to promote FGF signaling in Xenopus embryos. It is unclear whether FLRT3 regulates FGFR signaling directly or via the recruitment of cytoplasmic proteins; however, the cytoplasmic domain of FLRT3 seems to be necessary for signaling (Bottcher, Pollet et al. 2004). Conversely, the extracellular LRR sequence has been shown to be essential to homotypic cell sorting in FLRT3-positive HEK293T and Xenopus embryos whereas the intracellular portion was not essential to this process (Karaulanov, Bottcher et al. 2006). Further on, FLRT3 was shown to regulate cell adhesion, acting synergistically with UNC5b to induce cell de-adhesion by acting downstream on the Rho small GTPase Rnd1. Interaction of the
FLRT3 cytoplasmic tail with Rnd1 leads to a decrease in C-cadherin levels, which in turn leads to decreased cell-cell contacts (Ogata, Morokuma et al. 2007, Karaulanov, Bottcher et al. 2009). Overexpression of FLRT3 in Xenopus embryos can even lead to cell dissociation; however, paraxial protocadherin (PAPC) forms a complex with FLRT3 to regulate its de-adhesion properties by inhibiting the recruitment of Rnd1 (Chen, Koh et al. 2009). The interaction between FLRT3 and UNC5d is dependent on the LRR sequence of FLRT3, while the downstream effects on Rnd1 are dependent on the intracellular portion of the protein (Ogata, Morokuma et al. 2007, Karaulanov, Bottcher et al. 2009). It appears that FLRT3 may have a bifunctional role in cell adhesion since it is able to promote both homotypic cell sorting and cell-deadhesion. The expression of FLRT3 in the developing brain implies that it is involved in neural development (Haines, Wheldon et al. 2006, Gong, Mai et al. 2009). In line with this, it has been shown that FLRT3 promotes neurite outgrowth both non-cell autonomously and cell autonomously (Robinson, Parsons Perez et al. 2004, Tsuji, Yamashita et al. 2004). Similar to FLRT2, the shed FLRT3 ECD was shown to negatively regulate the migration of cortical neurons in cooperation with the UNC5b cell-surface receptor (Yamagishi, Hampel et al. 2011).

1.8 Research Model: The ATDC5 Cell Line

The ATDC5 cell line was derived from a mouse teratocarcinoma (Atsumi, Miwa et al. 1990). It is a pre-chondrogenic cell line that remains undifferentiated when cultured in regular medium. When grown in the presence of insulin or insulin-like growth factor-1, these cells are able to replicate the events of chondrogenesis, from cell condensation through to cartilage nodule formation (Shukunami, Shigeno et al. 1996, Shukunami, Ohta et al. 1998). The ability of the ATDC5 cells to undergo chondrogenesis has made it an invaluable model with which to study the cellular and molecular aspects of chondrogenesis. Xu et al. (2011) showed that the ATDC5 cell line expresses FLRT2 at both the mRNA and protein level (figure 1-4). The endogenous
presence of FLRT2 in the ATDC5 cell line allows one to analyze and characterize the expression and function of FLRT2 in a more natural environment where the protein is normally expressed. This is in contrast to many of the previous studies that utilized FLRT2 transfected cell lines, a strategy that could potentially result in conclusions that are not representative of the in vivo state. Protein interactions underlying adhesion, condensation, and chondrogenesis are complex and involve the orchestration of many molecules. The cellular environment in which a protein is expressed may greatly alter its properties and/or function.

Figure 1-4: Temporal Expression of FLRT2 in the ATDC5 cell line during chondrogenesis. Western blots to detect FLRT2 were conducted with lysates from ATDC5 cells grown in maintenance medium (P) or in the presence of insulin to stimulate chondrogenic differentiation (harvested at days 0, 4, 6, 10, and 14). Reprinted from Xu et al, with permission from John Wiley and Sons.

Although the ATDC5 cell line provides and elegant model for the study of FLRT2 in relation to condensation and chondrogenesis, it does not come without limitations. Like with any in vitro system, there is the possibility that the model does not truly represent what occurs in vivo. Being an immortalized cell line it inherently has mutations that could potentially have effects on the processes being studied. Likewise, cells grown on 2D planar substrates are not always completely representative of what occurs in a 3D setting (Cukierman, Pankov et al. 2001).
1.9 **Significance of Study**

During craniofacial development, CNCC migrate from the mid- and hind-brain to the developing frontonasal area and branchial arches. Here they participate in epithelial-mesenchymal interactions that precede cellular condensation, which is required to initiate cellular differentiation that leads to histogenesis. Studies have demonstrated that FLRT2 is expressed in key areas during development: along the migration pathways of CNCC, at areas of epithelial-mesenchymal interactions, and in the developing branchial arches and frontonasal process where condensations will result in the formation of cartilage and bone (Gong, Mai et al. 2009). This distinct spatial and temporal expression data suggests that FLRT2 plays a very important role during many key stages of craniofacial development. Exactly where, and how this protein contributes is therefore worthy of investigation.

1.10 **Objectives**

The expression and function of FLRT2 was investigated in the ATDC5 pre-chondrogenic cell line. More specifically, the objectives of this study were:

1) To determine the sub-cellular localization of FLRT2 in the ATDC5 cell line

2) To identify any FLRT2 protein in ATDC5-derived ECM

3) To study FLRT2 expression in relation to other ECM proteins (in particular, fibronectin)

1.11 **Hypothesis**

Based on its structural characteristics, expression profile, and previous research results, it was hypothesized that in ATDC5 chondroprogenitor cells, FLRT2 interacts with the ECM.
Chapter 2 - Materials and Methods

2 Materials and Methods

2.1 Cell Culture

ATDC5 cells (ATCC) were cultured in maintenance medium consisting of Dulbecco’s Modified Eagle Medium: Nutrient Mixture F12 (DMEM:F12) supplemented with 5% fetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin, and 250ng/ml amphotericin B (Invitrogen). Cells were incubated in a 37°C incubator in 100% humidity and 5% CO₂.

2.2 Antibodies and Peptides

Primary antibodies: A mouse monoclonal antibody recognizing an extracellular epitope of FLRT2 (R&D systems) was used in immunofluorescence studies. A goat polyclonal antibody raised against a peptide representing the extracellular portion of FLRT2 (R&D systems) was used in the immunoprecipitation/immunoblotting experiments. For detection of fibronectin, a rabbit polyclonal anti-fibronectin antiserum (Sigma) was employed in both immunofluorescence and immunoprecipitation/immunoblotting experiments. Normal rabbit IgG (Cell Signaling) was used as an isotype control in immunoblotting.

Secondary antibodies: Detection of the mouse anti-FLRT2 antibody was conducted with Alexa-488 and Alexa-594 tagged goat anti-mouse polyclonal antibodies (both Invitrogen). The goat anti-FLRT2 polyclonal antibody was detected with a horseradish peroxidase conjugated donkey anti-goat secondary antibody (Cell Signaling). The anti-fibronectin antiserum was detected with an Alexa-633 tagged goat anti-rabbit polyclonal antibody (Invitrogen) in immunofluorescence experiments, or a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Cell Signaling) in immunoprecipitation/immunoblotting experiments.
**Peptide:** A peptide representing the Cys36-Ser539 amino acid residues of FLRT2 was used in both the immunofluorescence and immunoblotting experiments. This sequence corresponds to the majority of the extracellular portion of FLRT2.

### 2.3 Immunolocalization of FLRT2

ATDC5 cells were seeded at a density of 1.5X10^5 per well into 96 well, 0.17mm glass-bottom plates (Matrical Bioscience) and incubated for 16 hours before immunostaining was conducted.

**Live cell staining:** Culture medium was removed from culture wells and cells were blocked with 5% goat serum (Gibco) in DMEM:F12 for 30 minutes on ice. Blocking medium was removed and replaced with primary antibody solution containing 10µg/ml mouse anti-human FLRT2 antibody (R&D systems) in DMEM:F12. Primary antibody incubations were conducted on ice for 1 hour. Cells were rinsed 3 times, 5 minutes each, with ice-cold phosphate buffered saline (PBS), followed by fixation in 4% paraformaldehyde (Sigma) at room temperature for 15 minutes. Cells were again washed three times with ice-cold PBS, followed by incubation with secondary antibody solution containing Alexa488-tagged polyclonal anti-mouse IgG (1 µg/ml, Invitrogen) and Hoechst 33342 nuclear stain (1 µg/ml, Invitrogen) in DMEM:F12. Cells were incubated with secondary antibody for 60 minutes at room temperature, followed by 3 washes with ice cold PBS. 100µl of PBS was added to each well and cells were visualized using confocal microscopy.

**Negative controls:** Four controls were used to verify the specificity of the mouse anti-FLRT2 antibody staining and to set appropriate image acquisition parameters (see below). First, an isotype-matched (IgG2b) mouse IgG (10 µg/ml, Abcam) was used in place of primary antibody in order to confirm that there was no non-specific binding of the primary antibody to potential IgG receptors present on the surface of the cells. Second, an alternate anti-mouse IgG secondary
antibody tagged with Alexa-594 (1µg/ml, Invitrogen) was used in order to rule out whether the signal generated in the Alexa-488 range was due to cellular autofluorescence. Third, DMEM:F12 alone was used in place of the primary antibody solution in order to observe any non-specific binding of the secondary antibody. Lastly, the mouse anti-FLRT2 primary antibody was pre-incubated with a 5-fold molar excess of FLRT2 peptide (R&D systems) in order to saturate the binding sites on the antibody. The antibody:peptide preparation was incubated overnight at 4°C and subsequently centrifuged for 20 minutes at 16,000g. The supernatant was removed, diluted to 10µg/ml in DMEM:F12, and used in place of primary antibody.

**Fixed cell staining:** Medium was removed from culture wells and cells were fixed in 0.5% paraformaldehyde at room temperature for 30 minutes. Cells were subsequently washed 3X in ice cold PBS and incubated with primary antibody solution (as above) for 1 hour at room temperature. The cells were again washed 3X with ice cold PBS and incubated with secondary antibody (as above) for 1 hour at room temperature. After a final 3 washes in PBS, cells were reconstituted in 100µl PBS and visualized using confocal microscopy.

**Fixed/permeabilized cell staining:** Protocol was the same as for fixed cell staining, with the addition of 0.01% saponin (Sigma) to primary and secondary antibody solutions.

**Image acquisition:** Images were acquired with an Olympus IX81 inverted fluorescence microscope equipped with a Hamamatsu C9100-13 EM-CCD (SickKids Imaging Facility). Acquisition parameters (exposure time, laser intensity, and sensitivity) for each fluorescent channel were set based on the negative control samples (mouse IgG2b and secondary antibody alone) so that these images showed no background fluorescence. Acquired 2D images were processed and compiled using the ImageJ software program. 3D images were reconstructed with Volocity (PerkinElmer).
2.4 ECM Staining

**Generation of ATDC5-derived ECM:** Ninety-six well glass-bottom matriplates were pre-coated with 10µg/ml fibronectin (Sigma). Briefly, 100µl of fibronectin (diluted in PBS) was added per well and plates were incubated overnight at room temperature. The solution was removed, wells were washed 2X with PBS, and the plate was subsequently used for cell culture. ATDC5 cells were seeded at a density of 2X10^6 cells/well and incubated in a 37°C incubator in 100% humidity and 5% CO₂ for 7 days after reaching confluence (about 9 days total). Culture medium was changed every 2 days.

**Cell extraction/harvesting of ATDC5-derived ECM:** At 7 days post-confluency, ATDC5 cells were extracted from cultures by removing culture media and placing 100 µl/well of extraction solution containing 20mM NH₄OH (Sigma) and 0.5% (v/v) Triton X-100 (Sigma) in PBS, prewarmed to 37°C. After 5 minutes, removal of cells was verified by visualization under a microscope. The extraction solution was removed and the remaining ECM was washed 5X with PBS. The harvested ECM was used immediately in the immunostaining protocol.

**Immunostaining:** Both the intact post-confluent ATDC5 cell cultures and the ATDC5-derived ECM were stained according to the live cell staining protocol described above. The primary antibody solution contained both the anti-FLRT2 antibody and a rabbit anti-human fibronectin antiserum (Sigma). The secondary antibody solution contained Alexa488-tagged goat anti-mouse IgG, Alexa633-tagged goat anti-rabbit IgG, and Hoescht 33342 nuclear stain (all 1µg/ml, Invitrogen). Some samples also contained Alexa594-tagged phalloidin (1:30,000U/mL, Invitrogen) to visualize F-actin in intact cell cultures and to ensure the complete removal of cell bodies in the ECM preparations.
2.5 ATDC5 Co-incubation with Fibronectin Coated Microbeads

Polycarboxylate modified beads (2µM, Polysciences) were resuspended in 10µg/mL fibronectin (Sigma) or 100 µg/ml poly-L-lysine (PLL, Sigma) and incubated at 37°C for 1 hour. Bead solutions were then centrifuged at 12,000 rpm for 5 minutes and the resulting supernatant was removed. The beads were then resuspended in 1mL of PBS, sonicated for 5 seconds, and counted using a hemocytometer. ATDC5 cells were seeded into wells of a 96-well glass-bottom matriplate at a density of 2X10^6 cells/well and incubated for 16 hours at 37°C. Cells were briefly removed from the incubator and either fibronectin- or PLL-coated beads were added to cells at a volume equivalent to 100,000 beads per well (5 beads/cell). Cells/beads were incubated for an additional 3 hours at 37°C and then live cell immunostaining was conducted using the methods described above.

2.6 Blocking Fibronectin Fibril Formation

In order to prevent the extracellular assembly of fibronectin fibrils, ATDC5 cells were seeded at a density of 2X10^6 cells/well in culture medium containing the 70kDa N-terminal fragment of fibronectin (70 µg/mL, Sigma) or vehicle alone (H2O). Cells were cultured for either 2 or 7 days. In the 7 day cultures, culture medium (containing the fibronectin fragment or vehicle) was changed on day 4.

Immunostaining for FLRT2 and fibronectin was conducted on days 2 and 7 according to the usual live cell protocol. In some samples, FLRT2 and fibronectin were stained separately in order to ensure that the fluorescent signal in the Alexa488 range was not a result of overspill from a strong signal in the Alexa633 range.
2.7 Co-immunoprecipitation Experiments

Immunoprecipitation: ATDC5 cells were washed with PBS and cells were harvested by scraping. Cells were pelleted by centrifugation at 1500 rpm for 5 minutes. Cells where then resuspended and lysed in 1X RIPA [20 mM Tris-HCl (pH 7.5); 0.15 M NaCl, 1 mM Na$_2$EDTA; 1 mM EGTA; 1% NP-40; 1% sodium deoxycholate; 2.5 mM sodium pyrophosphate; 1 mM b-glycerophosphate; 1 mM Na$_3$VO$_4$; 1 µg/ml leupeptin, Cell Signaling Technology) containing 1mM phenylmethylsulfonyl fluoride (Sigma). Lysates were homogenized by brief (5 seconds) sonication. The resulting homogenates were centrifuged for 20 minutes at 14,000 g and 4°C. The supernatant was collected and protein concentration quantitated using the Bradford dye-binding method (Bio-Rad). Supernatants equivalent to 500 µg of protein were then combined with either mouse anti-FLRT2 polyclonal antibody (4 µg total, R&D systems) or rabbit anti-fibronectin antiserum (4 µg total, Sigma) and mixed overnight at 4°C. Immunocomplexes were captured with Protein A/G-agarose beads (CalBiochem) for 2 hours at 4°C. Beads were collected by pulse centrifugation and washed 1X in ice-cold RIPA buffer.

Immunoblotting: Collected beads were resuspended in sample buffer and boiled for 5 minutes at 100°C. 20 µL of sample was loaded per well onto 7% polyacrylamide gels and was subject to electrophoresis under reducing conditions. 2µg of normal rabbit IgG in sample buffer was used as an isotype control. Electrophoresed proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare) and blocked for 1 hour in 2% skim milk (BioShop) in Tris-buffered saline containing 0.1%Tween 20 (BioShop). The membranes were incubated with anti-fibronectin antiserum (1:500, Sigma) overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (0.5µg/ml, Cell Signaling) for 1 hour at room temperature. Signal was detected using ECL™ Western blotting
detection reagents (GE Healthcare), according to the manufacturer’s protocol, and visualized on ChemiImager™ (BioRad). Membranes were then stripped in buffer containing glycine, SDS, and Tween-20 (pH 2.2), and re-probed with a goat anti-FLRT2 polyclonal antibody (1µg/ml, R&D systems) overnight at 4°C followed by horseradish peroxidase conjugated donkey anti-goat secondary antibody (2µg/ml, BioShop) for 1 hour at room temperature. Signal was detected as previously described.
3 Results

3.1 FLRT2 is Located Intracellularly, at the Cell Membrane, and Extracellularly

In order to determine the distribution of FLRT2 in ATDC5 cells, immunolocalization experiments were performed using a monoclonal antibody directed against an epitope located in the extracellular domain of FLRT2. The initial immunolocalization experiments were conducted following standard fixation protocols, i.e., incubating cells in 4% paraformaldehyde for 15 minutes at room temperature. This fixation protocol resulted in a relatively weak FLRT2 signal that appeared to be exclusively intracellular (figure 3-1, a), suggesting fixation-associated permeabilization of the cell membrane. Fixation with a gentler protocol of 0.5% PFA for 30 minutes did not show intracellular FLRT2 staining (figure 3-1, b). Subsequent permeabilization of fixed cells with saponin generated a very strong intracellular FLRT2 signal (figure 3-1, c), with punctate staining throughout the cytoplasm that was slightly more intense in the perinuclear area. Expression of FLRT2 at the cell membrane was not seen with any of these fixation/permeabilization protocols (figure 3-1 a-c).
Figure 3-1 (a-c): Immunofluorescent localization of FLRT2 (green) in fixed ATDC5 cells. Cells fixed with 4% PFA for 15 minutes show some intracellular FLRT2 staining (a). Cells treated with a milder fixation protocol of 0.5% PFA for 30 minutes did not produce any FLRT2 signal at all (b). Saponin permeabilization of fixed cells resulted in a robust intracellular FLRT2 signal (c). None of these cell preparation methods produced FLRT2 staining at the cell membrane or in the ECM.

Since FLRT2 is a putative membrane protein, it was suspected that the presence of FLRT2 in only the intracellular component of fixed and fixed/permeabilized cells may indicate the destruction of the extracellular epitope of FLRT2 with PFA fixation. Live cell imaging was next performed, fixing cells after the incubation with primary antibody. These experiments revealed that the FLRT2 protein is in fact located along the cell membrane (figure 3-2, a). Comparison to the localization of F-actin shows that, in some instances, FLRT2 staining also appears to extend away from the cell membranes and is polarized to areas where cells are in close contact (asterisk, figure 3-2, b). In areas where cells are more confluent, the FLRT2 staining pattern takes on both of these characteristics, creating a web-like pattern between and around cells (figure 3-2, c-d).
Figure 3-2 (a-d): Immunofluorescent localization of FLRT2 (green), actin (red) and DNA (blue). FLRT2 appears to be localized at the cell membrane (a), but staining is also seen extending between cells (white asterisk, b). Images c-d show a combination of membrane-type and intercellular-type FLRT2 staining, creating a web-like pattern around and between the cells.

Reconstructed 3D images show that, in addition to the previously described staining pattern, FLRT2 is also present along the base of the culture surface in some areas where cells are absent (white arrows, figure 3-3, a and c). In comparison, the mouse IgG2b negative control sample
reveals no signal (figure 3-3, b and d), suggesting that the FLRT2 staining along the base of the plate is associated specifically with the anti-FLRT2 antibody.

**Figure 3-3 (a-d):** Reconstructed 3D images of samples stained with an anti-FLRT2 antibody (a,c) and negative control Mu IgG<sub>2b</sub> (b,d). Note the FLRT2 signal along the base of the culture surface (white arrows; a,c) in areas devoid of cells. The mouse IgG<sub>2b</sub> negative control does not show the same type of staining pattern. Alternate views (images rotated to show side view) of FLRT2 (c) and Mu IgG<sub>2b</sub> (d) demonstrate FLRT2 staining at the base of the culture surface.

Specificity of the FLRT2 antibody was confirmed with the use of appropriate controls. Cells that were treated with a non-specific Mouse IgG<sub>2b</sub> antibody in place of the mouse anti-FLRT2 antibody did not generate any signal (figure 3-3, b-d). When the anti-FLRT2 peptide was pre-incubated with the FLRT2 peptide and subsequently used to stain cells, the usual extracellular
and cell-membrane staining pattern was abolished (figure 3-4, a). The use of an alternately-tagged (Alexa594) secondary antibody to stain detect FLRT2 yielded a signal and staining pattern that was comparable to that with the use of the Alexa 488 conjugated secondary antibody (figure 3-4, b)

Figure 3-4 (a-b): Controls to confirm specificity of FLRT2 staining. Cells were treated with anti-FLRT2 antibody saturated with FLRT2 peptide, resulting in a loss of the usual FLRT2 signal (a). Alternately, an Alexa 594-tagged secondary antibody was used to rule out autofluorescence (in the 488nm range) as the source of signal (b).

3.2 FLRT2 is Located in the ATDC5-derived ECM

The data so far suggested the possibility that FLRT2 was present in the ECM. To investigate the possible distribution of FLRT2 in the ECM, cells were grown for 7 days post-confluency to allow for the production and maturation of ECM. Intact, live cell cultures were immunostained as usual to visualize FLRT2, actin, and fibronectin (a marker for the ECM).
Strong fibronectin staining (figure 3-5 c-d) confirmed that the ATDC5 cells were secreting, assembling, and organizing ECM proteins. The position of the ECM that surrounds cells is denoted by the relative distribution of fibronectin and actin (figure 3-5 c-d vs e-f). Areas of intense FLRT2 staining correlate strongly with areas of fibronectin staining, suggesting that FLRT2 may be located in the ECM (figure 3-5 a-b vs c-d). Although FLRT2 staining does appear to be present in some areas where actin is evident, the signal here is much less intense (figure 3-5 a-b vs e-f). Additionally, the appearance of extra-nuclear structures that fluoresce in the same range as Hoechst 33342 can be noted (figure 3-5, white arrows). These are located in the ECM and have a fibril-like appearance, suggesting that they may represent matrix fibers.
Figure 3-5 (a-h):
Immunolocalization of FLRT2, fibronectin, and actin in post-confluent ATDC5 cultures. FLRT2 = green, fibronectin = purple, actin = red, DNA = blue. Note that FLRT2 staining (a, b) overlaps with fibronectin staining (c, d) in many areas, while there is less colocalization with actin (e, f). Merged figures (g, h) show the relative localization of all three proteins. There are extra-nuclear structures that are fluorescing in the same range as the Hoechst 33342 nuclear stain. This may represent an autofluorescent ECM protein. White arrows (a,c,e) denote an area of FLRT2 staining that is independent of both actin and fibronectin staining. Here, FLRT2 is colocalized with the unidentified autofluorescent putative ECM protein.
To further investigate whether FLRT2 is present in the ECM, and if so, whether it colocalizes with fibronectin, experiments were conducted on the ATDC5-derived ECM after cell removal with NH₄OH and Triton X-100 (Figure 3-6). Confirmation of cell removal in the cultures was obtained by the observation of a lack of actin and nuclear staining (figure 3-6 e-f) and the presence of fibronectin staining surrounding spaces formerly occupied by cells (figure 3-6 c-d). FLRT2 staining is evident throughout the ECM, although the intensity varies. Similar to the previous live cell staining results, areas of intense FLRT2 staining colocalize with areas of more intense fibronectin staining (figure 3-6 a-b). To confirm that the high degree of observed colocalization between FLRT2 and fibronectin was not an imaging artifact resulting from the Alexa633 fluorophore generating a signal at two different wavelengths, experiments also included samples stained only for fibronectin and imaged during excitation at both 491nm (Alexa 488 range) and 642nm (Alexa 633 range). Though the fibronectin signal generated via excitation at 642nm is very intense, excitation of the same sample with the 491nm laser does not produce any signal (figure 3-7, a-b), confirming that the colocalization of the respective FLRT2 and fibronectin signals truly represents a colocalization of these proteins. Thus, the localization of FLRT2 in the ATDC5-derived ECM after cell extraction corroborates the observations from intact cell cultures – FLRT2 and fibronectin are highly colocalized.
Figure 3-6 (a-f): Immunolocalization of FLRT2, fibronectin, and actin in the ATDC5-derived ECM. FLRT2 = green, fibronectin = purple, actin = red, DNA = blue. FLRT2 staining is clearly evident in the remaining ECM (a-b). Intensity of FLRT2 expression seems to correlate somewhat with the intensity of fibronectin staining (c-d). Merged images fail to show actin staining, confirming cell extraction (e-f).
**Figure 3-7 (a-b):** Fibronectin staining alone (with an Alexa633-tagged secondary antibody) shows a robust signal when excited at 642nm (a). There is no signal generated from the same sample when excited at 491nm (b), confirming that the observed colocalization of FLRT2 and fibronectin is not the result of a single fluorophore being excited at dual wavelengths. Note here again the presence of autofluorescent extracellular structures excited at the same wavelength as the Hoechst 33342 nuclear stain.

### 3.3 FLRT2 Interacts With Fibronectin-coated Microbeads

Next, the ability of FLRT2 to interact with fibronectin-coated beads was tested. ATDC5 cells were incubated with fibronectin-coated polycarboxylate-modified 2μM beads and subsequently stained to identify FLRT2 and fibronectin. Bright field images reveal the location of beads in both the experimental and control (PLL-coated) bead samples (figure 3-8, a-b). Fibronectin staining in the experimental samples confirms that the beads are coated with fibronectin (figure 3-8, e) whereas both the control and experimental samples demonstrate the formation of extracellular fibronectin fibrils (figure 3-8, e-f). FLRT2 staining in the experimental samples repeats the previously-observed colocalization of FLRT2 with extracellular fibronectin fibrils.
Additionally, FLRT2 staining is observed in association with a considerable proportion of the fibronectin-coated beads (white arrows, figure 3-8), indicating that FLRT2 is interacting (either directly or indirectly) with these beads. The negative control samples fail to show FLRT2 staining around the PLL-coated beads (figure 3-8 b,d,f), confirming that this pattern is specific to the fibronectin coating.
Figure 3-8 (a-f): FLRT2 and fibronectin staining of ATDC5 cells co-incubated with fibronectin- and PLL-coated microbeads. FLRT2 = green, fibronectin = purple, DNA = blue. Bright field images show the location of fibronectin-coated (a) and PLL-coated (b) beads. Cells that were incubated with fibronectin coated beads (a,c,e) show FLRT2 staining colocalized with fibronectin fibrils and around fibronectin-coated beads (denoted by white arrows). Cells that were incubated with PLL-coated beads (b,d,f) show FLRT2 staining in the same areas as fibronectin, however, bead-associated FLRT2 staining is absent.
3.4 The Localization of FLRT2 is Altered When Fibronectin Fibril Formation is Blocked

In order to investigate the relationship between FLRT2 and fibronectin matrix formation, ATDC5 cells were incubated with the 70kDa N-terminal portion of fibronectin with the purpose of blocking fibronectin fibril formation. Negative control samples were cultured with an equivalent amount of vehicle only (H₂O).

3.4.1 2 Day Cultures

On day two, cultures demonstrated an almost complete inhibition of fibronectin fibril formation (figure 3-9, a). A small degree of fibronectin staining is visible, but this is uncharacteristically weak and restricted to the cell membrane, likely representing secreted fibronectin that is bound to cell-surface receptors but that cannot self-associate to form fibrils. FLRT2 staining in this sample is affected in a parallel manner, demonstrating a much weaker signal that is restricted to the surface of the cell (figure 3-9, b). Conversely, the negative control sample that was incubated with vehicle only shows a typical extracellular fibronectin fibril staining pattern and a colocalized FLRT2 staining pattern (figure 3-9, c and d). This validates that the observed decrease in FLRT2 signal is indeed a result of incubation with the 70kDa N-terminal portion of fibronectin.

3.4.2 7 Day Cultures

Continued blockage of fibronectin fibril formation over the course of 7 days results in a partial rebound in fibronectin fibril formation in experimental samples (figure 3-10, a). The culture medium containing the 70kDa N-terminal fragment of fibronectin or vehicle alone was refreshed on day 4, but long term culture permitted some degree of fibril formation. Comparison of the appearance of fibronectin staining in experimental and control samples suggests a higher degree
of maturation and reorganization in the vehicle only samples (figure 3-10, a-b). Cultures containing the vehicle only exhibit a more mesh-like pattern suggestive of a network of fibers, whereas cultures containing the fibronectin fragment contain fibrils that are more solitary and, in most areas, more closely resemble those that form after 2 days under normal conditions.

Concomitant with the reappearance of fibronectin fibrils, FLRT2 staining in the 7-day experimental samples is also present (figure 3-10, c). Comparison to the vehicle-only control sample, however, reveals that the distribution and intensity of FLRT2 staining is greatly decreased in cultures grown in the presence of the 70kDa N-terminal region of fibronectin (figure 3-10, d). The vehicle only culture shows much more widespread FLRT2 staining. Interestingly, FLRT2 and fibronectin are highly colocalized in the experimental samples (figure 3-10, e) while in the vehicle only control there is a significant amount of FLRT2 staining that is independent of fibronectin (figure 3-10, f).
Figure 3-9 (a-d): Effects of 2-day incubation with the 70kDa N-terminal portion of fibronectin. FLRT2 = green, fibronectin = purple, DNA = blue. Fibronectin staining of experimental samples shows a relatively weak signal that is restricted to the cell membrane (a). Conversely, the vehicle alone control sample demonstrates the typical fibronectin fibril pattern extending between cells (c). Blocking of fibronectin fibril formation resulted in a concomitant decrease in FLRT2 signal (b), while a robust FLRT2 signal that is colocalized with fibronectin is seen in the vehicle-only control (d).
Figure 3-10 (a-d): Effect of 7-day incubation with the 70kDa N-terminal portion of fibronectin. Experimental samples show a re-emergence of fibronectin fibril formation (a), though fibrils have a less mature appearance than those in the vehicle-only control (b). FLRT2 staining in the experimental sample is present and highly colocalized with fibronectin fibrils (c). The vehicle-only control reveals an intense FLRT2 signal that suggests an accumulation of the protein in the ECM, and is less co-localized with fibronectin (d). Merged images show the different degrees of colocalization of FLRT2 and fibronectin in the experimental (e) and control (f) samples.
3.5 Co-Immunoprecipitation of FLRT2 and Fibronectin

To test whether FLRT2 and fibronectin interact at a biochemical level, ATDC5 cell lysates were immunoprecipitated with either anti-FLRT2 or anti-fibronectin antibodies. Co-immunoprecipitation of cell lysates with FLRT2 and fibronectin revealed interactions of these two proteins: immunoblotting of FLRT2 and fibronectin precipitates with anti-fibronectin antiserum and an anti-FLRT2 antibody, respectively, revealed bands corresponding approximately to the predicted molecular weights of both proteins (figure 3-11). This co-immunoprecipitation experiment lends further evidence for the interaction of FLRT2 and fibronectin in ATDC5 cells.
Figure 3-11: Immunoprecipitation and immuno blotting of ATDC5 cell lysates. ATDC5 cell lysates were subject to immunoprecipitation with anti-FLRT2 (FLRT2-IP) or anti-fibronectin (Fibro-IP) antibodies. Immunoprecipitates were run on gels alongside the FLRT2 peptide, ATDC5 cell lysates, and normal rabbit IgG and then transferred to membranes. The membrane was probed with anti-fibronectin antibody, showing bands corresponding to the molecular weight of fibronectin in the ATDC5 lysates, FLRT2 immunoprecipitates, and fibronectin immunoprecipitates, but not in the FLRT2 peptide sample (blot on left). Arrowheads represent the upper (270kDa) and lower (220kDa) molecular weight range of possible fibronectin isoforms. Re-probing with an anti-FLRT2 antibody revealed bands in all four samples, migrating at approximately the same molecular weight (blot on right).
Chapter 4 - Discussion

4 Introduction

The molecular role of the FLRT2 protein has previously been investigated in various in vitro models. Most of the research conducted to date has employed transfected cell lines that normally display little to no endogenous expression of FLRT2. In these cases, FLRT2 has been characterized mainly as a transmembrane protein with a focus on its putative roles in cell adhesion and cell signaling in partner with other cell-surface molecules such as FGF receptors. More recently, however, FLRT2 has been studied in endogenously-expressing primary mouse neurons and was proven to exist in an extracellular form that is cleaved and shed into the surrounding ECM where it remains active in guiding neuronal cell migration (Yamagishi, Hampel et al. 2011). This opens up entirely new possibilities in terms of the functions of FLRT proteins. In light of this evidence, it is important to consider the impact that cellular phenotype and the surrounding environment may have on which form FLRT2 will exist and its accompanying function. Therefore, the study of FLRT2 in a more native environment, such as that of the endogenously expressing ATDC5 cell line, is beneficial since it will more accurately represent in vivo conditions. This promises to offer new insight into the form, location, and function of the protein.

4.1 Localization of FLRT2 in ATDC5 cells

As the ATDC5 cell line was generated from a mouse embryonal carcinoma, the use of a murine primary antibody was of concern. Proper controls were therefore used in this study to confirm that the observed FLRT2 staining is specific. The presence of any Fc receptors on the cell surface, although unlikely based on the cell type, may be a cause of non-specific binding of
primary antibody. Treatment of cells with an isotype-matched (IgG2b), non-specific mouse monoclonal antibody was used to rule this out. The lack of signal in these samples confirms that this is not the case. Additionally, cells treated with a solution of anti-FLRT2 antibody that was pre-incubated with an excess of FLRT2 peptide (from the same manufacturer as the antibody) fail to show an appreciable signal, further substantiating the claim that the observed FLRT2 staining pattern is specific.

The immunolocalization of FLRT2 at the cell membrane in sub-confluent ATDC5 cell cultures corroborated the findings of Haines et al (2006) with transfected COS-7 cells. In areas of greater cell confluence, however, FLRT2 appears to extend between cells in a web-like pattern away from the terminal ends of F-actin (delineating approximately the boundary of the plasma membrane), suggesting the possibility that FLRT2 is present extracellularly in addition to being at the cell membrane. Interestingly, paraformaldehyde fixation of cells results in ablation of the typical extracellular FLRT2 staining pattern while permeabilization of pre-fixed cells demonstrates that intracellular staining is unaffected by fixation. There are a few possibilities as to why this might be occurring. The FLRT2 antibody is a monoclonal and fixation might result in the masking of the epitope via paraformaldehyde-associated crosslinking of lysine residues either within or near the site of antibody recognition. However, the presence of intracellular staining in fixed and permeabilized cells does not necessarily support this hypothesis since fixation would be expected to affect intracellular antigen in the same manner. Another possibility is that FLRT2 possesses a different 3-dimensional conformation as it is being trafficked intracellularly compared to the protein on the cell surface, resulting in a different degree of epitope susceptibility to fixation. Yet another possibility for the absence of extracellular FLRT2 signal concomitant with PFA fixation is that FLRT2 may be closely interacting with either a cell-surface or an extracellular protein. Thus, fixation could cross-link
these two proteins together, and in doing so prevent the antibody from accessing the appropriate epitope. In this case, intracellular FLRT2 would not likely be affected as it would not yet be available to interact fully with other proteins. Unfortunately, the exact epitope of the antibody used is not known (proprietary information, R&D systems), with the accompanying literature stating that a peptide representing the Cys36-Ser539 residues (the majority of the extracellular domain of FLRT2) was used to generate the antibody. As a result, the analysis of amino acid sequence near the epitope cannot be conducted and the possibility of lysine cross-linking cannot be ruled out as the cause of signal loss. Binding experiments using a library of FLRT2 deletion mutants would be required to determine the epitope, a study beyond the scope of this work. It would be interesting to repeat the immunofluorescence experiments with either a polyclonal anti-FLRT2 antibody or an alternate monoclonal antibody to see how fixation affects the staining pattern when other epitopes are recognized.

4.2 FLRT2 May be Cleaved From the Surface of ATDC5 Cells

The collective data suggest that FLRT2 is cleaved in ATDC5 cells. In sub-confluent ATDC5 cultures, FLRT2 staining is present in areas devoid of cells, suggesting that FLRT2 is being cleaved at the cell surface with the shed portion then sticking to the surface of the plate. The cell cultures were grown in the presence of fetal bovine serum (FBS). This contains numerous proteins that can form a pseudo-matrix type coating along the base of the plate, providing a substrate for FLRT2 binding. It is interesting to note that FBS contains fibronectin, which has been shown to be incorporated into the endogenously derived matrix of cells in culture and may therefore also be binding to the culture surface (Hayman, Ruoslahti 1979). FLRT2 immunolocalization in post-confluent cultures provides further evidence in support of the idea that FLRT2 is cleaved from the cell surface. FLRT2 appears to be partially associated with the cell membrane (i.e. in areas adjacent to actin), though this signal is relatively weak compared to
the much more robust extracellular signal. Here the staining pattern is suggestive of an accumulation of protein in the ECM as the distribution is much more widespread than what would normally be seen with staining at the cell membrane. The partial colocalization of FLRT2 with fibronectin, an ECM protein, suggests again that FLRT2 is being shed from the cell surface and accumulating in the ECM. The presence of FLRT2 staining in the ATDC5-derived ECM after cell extraction may also substantiate this. The FLRT2 that is left behind in the ECM may represent the *cleaved* portion of the protein. This staining, however, could also be a product of membrane-bound FLRT2 interacting with an ECM protein and thus being left behind after detergent solubilization of cell membranes.

Preliminary western blot data have given hints that FLRT2 is being cleaved from the surface of ATDC5 cells (see figure 1-4). Immunobloting of ATDC5 cell lysates with the mouse monoclonal anti-FLRT2 antibody repeatedly revealed a strong band migrating at around 75kDa (Xu, Wei et al. 2011). This corresponds more closely to the predicted size of the *unglycosylated* full-length FLRT2 (74kDa), compared to the 85-100 kDa glycosylated forms shown in various cell lines (Haines, Wheldon et al. 2006, Yamagishi, Hampel et al. 2011, Lacy, Bonnemann et al. 1999). The 75kDa band observed in ATDC5 cells also corresponds closely to the weight of the extracellular, shed FLRT2 fragments described by Yamagishi *et al* (2011). A similar band size is seen in the immunoprecipitation experiments where blots were probed with an alternate polyclonal anti-FLRT2 antibody, verifying that this band is likely specific to FLRT2. Why then in ATDC5 cells would a band not be seen at 85-100kDa, representing the full-length transmembrane form of FLRT2? The ATDC5 cell line is prechondrogenic and will be eventually be heavily involved in matrix formation and reorganization. Thus, it can be postulated that these cells may already have a relatively high level of proteases that could potentially be involved in the cleavage of FLRT2. Nazakora *et al*. (2010), for instance, have
shown a robust expression of the protease ADAM10 in ATDC5 cells, which is involved in the cleavage N-cadherin from the surface of cells during chondrogenesis (Nakazora, Matsumine et al. 2010). It is possible that FLRT2 is cleaved very soon after it reaches the cell membrane. In this case, the main constituent of FLRT2 in cell lysates would be the cleaved form, which would account for the absence of the expected 100kDa band when cells are probed with an antibody against the extracellular portion of FLRT2.

The data suggesting that FLRT2 is cleaved in ATDC5 cells is supported in other studies with different cells. FLRT2-transfected COS-7 cell lysates produce a 20kDa band on western blots when probed with an antibody against the cytoplasmic region of the protein, likely representing the transmembrane/intracellular portion of FLRT2 that is left behind after cleavage of the ectodomain (Haines, Wheldon et al. 2006). Cell-conditioned media from primary mouse neurons and FLRT2-transfected HEK293T cells show two different FLRT2 fragments running at lower molecular weights than the full length protein found in cell extracts, indicating that the FLRT2 ectodomain is cleaved at two different sites and shed from the cell surface (Yamagishi, Hampel et al. 2011). Further experiments are required to substantiate the hypothesis that FLRT2 is being cleaved from the surface of ATDC5 cells. A comparison of the forms of FLRT2 present in complete ATDC5 cell lysates, microsomal membrane preparations, and cell-conditioned media would be useful in confirming the presence and relative amount of the cleaved form of FLRT2. Western blots with microsomal membrane preparations, which are enriched in plasma membrane proteins, would be expected to yield the membrane-bound, full-length FLRT2. Conversely, cell-conditioned media would be expected to yield the shorter, cleaved form of the protein. Complete cell extracts would be expected to continue to produce the different possible forms of FLRT2, i.e. the cleaved ectodomain and the intracellular/transmembrane domain (around 20kDa), depending on the epitopes recognized by the FLRT2 antibody that is used. If
FLRT2 is being cleaved shortly after it reaches the cell membrane, it is possible that the full-length protein might also be visualized in complete cell extracts. Because the relative amount of the full length FLRT2 at the cell surface would be low, an increased concentration of total protein would have to be loaded onto gels in order to visualize this band in western blots. If these experiments demonstrate that FLRT2 is indeed being cleaved from the surface of ATDC5 cells, it would be interesting to test the effect of various protease inhibitors to determine which type of enzymes that may be involved. Yamagishi et al (2011) demonstrated that the matrix metalloprotease/ADAM17 inhibitor TAPI-1 was able to prevent cleavage of the FLRT2 ECD in primary mouse neurons and transfected HEK293T cells. Similar enzymes may be involved in the putative cleavage of FLRT2 in ATDC5 cells.

Finally, there is a possibility that the molecular weight of FLRT2 is lower in ATDC5 cells due to a lack of glycosylation. Since the predicted molecular weight of FLRT2 based on its amino acid sequence is 74kDa, this would require an almost complete absence of glycosylation in ATDC5 cells (Lacy, Bonnemann et al. 1999). The fact that FLRT2 has been shown to be glycosylated in numerous cell types (SF9, COS-1, COS-7, HEK293T, and primary mouse neurons) makes this unlikely (Lacy, Bonnemann et al. 1999, Haines, Wheldon et al. 2006, Yamagishi, Hampel et al. 2011). Though this may seem like a remote possibility, it should be ruled out. This can be easily achieved by either: 1) treating cells with a glycosylation inhibitor such as tunicamycin, or 2) treating cell extracts with a deglycosylating enzyme such as N-glycosidase F. Resulting cell lysate preparations would be expected to show a shift towards a lower molecular weight form of FLRT2, confirming that the protein is indeed glycosylated in ATDC5 cells.
4.3 FLRT2 May be Involved in Cell-ECM Interactions

Previous research has implicated the FLRT proteins in cell adhesion. All three FLRT proteins co-localize with vinculin to cell-cell junctions in transfected COS-7 cells, while FLRT-transfected HEK293T and FLRT-injected Xenopus embryos demonstrate cell sorting that is dependent on the extracellular LRR domains (Haines, Wheldon et al. 2006, Karaulanov, Bottcher et al. 2006). FLRT3 also appears to control cell-deadhesion in cooperation with the UNC5b receptor and the cytoplasmic small GTPase Rnd1. Interaction of the FLRT3 cytoplasmic tail with Rnd1 leads to a decrease in C-cadherin, which in turn leads to decreased cell-cell contacts (Ogata, Morokuma et al. 2007, Karaulanov, Bottcher et al. 2009). Similar to the FLRT3-mediated decrease in C-cadherin seen in Xenopus embryos, increased FLRT2 expression in ATDC5 cells is associated with a decrease in N-cadherin (Xu, Wei et al. 2011). Both FLRT2 and FLRT3 are necessary for the maintenance of cell-cell contacts in the developing heart (Muller, Schulz et al. 2011). A lack of FLRT2 expression leads to loss of integrity of the epicardial sheath characterized by irregularities in cell-cell contacts and detachment from the underlying basement membrane. Thus, the FLRT proteins appear to have a dynamic role in mediating cell-cell adhesion, displaying almost contrasting effects (adhesion versus de-adhesion) in different environments.

This study provides evidence suggesting the involvement of FLRT2 in mediating cell-ECM adhesion, a role hinted at in previous studies of other members of the FLRT family. Cerebellar neurons produce longer neurites when plated on FLRT3-transfected Chinese hamster ovary (CHO) cells versus regular CHO cells (Tsuji, Yamashita et al. 2004). Similarly, FLRT3 expression in peripheral neurons is positively correlated with both neurite number and length (Robinson, Parsons Perez et al. 2004). In prechondrogenic (ATDC5) cells, FLRT2 expression correlates with the rate of cell migration into an artificially generated wound in the cell
monolayer, proposing the possibility that FLRT2 increases the rate of cell migration via an increase in cell-ECM interactions. In this study, the presence of FLRT2 staining in the ECM after cell extraction could signify one of two things: firstly, that FLRT2 interacts with ECM molecules, is cleaved from the cell membrane and is incorporated into or accumulating in the ECM; secondly, that FLRT2 interacts so strongly with ECM molecules that it is left behind after the cell membrane is solubilized. In either case, this suggests that FLRT2 may be interacting with the ECM.

This study shows that one of the possible ECM molecules with which FLRT2 may interact is fibronectin. It is interesting to note that the variations in intensity of FLRT2 staining in the ATDC5-derived extracellular matrix are almost identical to that of fibronectin staining. This may represent localized increases in the concentration of both of these proteins (implying co-localization) or may perhaps merely be an artifact of the 3-dimensional ECM being compacted into a more 2-dimensional form. This would cause certain regions of the collapsed ECM to overlap and appear more dense, causing a concomitant increase in fluorescent signal. The high degree of colocalization of FLRT2 and fibronectin in intact cell cultures corroborates the staining observed in the ECM, suggesting that the parallel variations in staining intensity represent a true colocalization of FLRT2 and fibronectin in the ECM.

A somewhat unexpected and serendipitous finding in the immunofluorescence images is the presence of autofluorescent structures that are distinct from cell nuclei. One possible explanation for this type of staining is the presence of extra-nuclear DNA associated with mycoplasma infection. The ATDC5 cells used in this study were tested for mycoplasma contamination (Charles River Laboratories) and were confirmed negative. Furthermore, the localization and shape of these structures is not what is typically seen with mycoplasma contamination. Their
fibrillar appearance and apparent extra-cellular location indicates that these structures represent autofluorescent extracellular matrix proteins. It has been previously documented that various cellular molecules and proteins fluoresce when excited at different wavelengths, and both collagen and elastin demonstrate autofluorescence when excited in the 350-400nm range (Wagnieres, Star et al. 1998). Confirmation that the autofluorescent fibers are indeed collagen or elastin fibers would require that cultures be stained with the appropriate antibodies. Unfortunately these were not available while staining was being conducted; however, this is definitely worthy of follow-up. In images of post-confluent ATDC5 cultures, FLRT2 is sometimes colocalized with the autofluorescent structures – and in some cases this occurs in areas where FLRT2 is not colocalized with fibronectin. The presence of FLRT2 staining in conjunction with autofluorescent putative ECM fibers that is independent of fibronectin staining is highly suggestive of the fact that FLRT2 is able to interact with other ECM proteins. While this data is quite preliminary, it does open up many interesting possibilities. Since we know that the ECM is a very complex structure composed of a diverse array of fibers and growth factors, it may undoubtedly require a great deal of effort to elucidate which of these is interacting with FLRT2.

Although the presence of FLRT2 in the ATDC5 derived ECM indicates a potential role in mediating cell-ECM interactions, it is still unclear whether FLRT2 is being incorporated into the matrix in the process. If western blot experiments indicate that FLRT2 is indeed being shed from the cell surface, it will be necessary to clarify how the cleaved ECD interacts with the matrix. In order to determine whether FLRT2 is actually being incorporated into the ECM, western blots to detect FLRT2 in deoxycholate (DOC) insoluble preparations of ATDC5-derived ECM can be performed. This would indicate whether FLRT2 is irreversibly integrated into the ECM and might provide clues to the strength of its interactions and thus its function here. Alternately, if
FLRT2 is found to be secreted into the ECM but it is not present in the DOC insoluble portion, this would indicate a more reversible interaction with the ECM and might suggest that FLRT2 is acting as a soluble factor that needs to retain the ability to dissociate from the ECM. Just as the components of the ECM are complex, the roles that they fill are extremely varied. Future work can be directed at characterizing the interactions of FLRT2 with the matrix and the functional significance of such interactions.

4.4 FLRT2 Interacts With Fibronectin

The data showing the strong colocalization of FLRT2 and fibronectin in ATDC5 cells and cell-derived matrix suggests that these proteins may be interacting. FLRT proteins closely resemble the small leucine rich ECM proteoglycans (e.g. decorin, biglycan, fibromodulin) that contain the same LRR motifs and have previously shown to be capable of interacting with other ECM molecules, including fibronectin (Mecham 2001). This study reveals that, in addition to colocalizing with fibronectin fibrils in ATDC5 cultures, FLRT2 localizes to cell-associated fibronectin-coated beads, in most cases surrounding the entire surface of the beads. Subsequent co-immunoprecipitation analysis with fibronectin and FLRT2 provides further evidence for an interaction between these proteins. Although the immunofluorescence and co-immunoprecipitation data show that FLRT2 and fibronectin are part of the same complex of proteins, they do not indicate whether these proteins are directly interacting. Direct interactions can best assessed with other techniques such as yeast two hybrid assays or fluorescence resonance energy transfer assays. Fibronectin has been shown to interact with various molecules, including other ECM proteins, cell surface receptors (e.g. integrins), GAGs, other fibronectin molecules, and bacteria and is therefore considered “sticky” (Mao, Schwarzbauer 2005, Singh, Carraher et al. 2010). Consequently, it is imperative to characterize the binding affinity between fibronectin and FLRT2 in order to determine the specificity of binding. An
ELISA-type binding assay using purified FLRT2 and fibronectin would be useful in determining the dissociation constant for the interaction between these two proteins. Comparison with known fibronectin-binders (e.g. integrin α5β1, fibrillin, fibromodulin, etc.) would provide an appropriate $K_d$ range for comparison.

4.5 Fibronectin Fibril Formation is Required for FLRT2 Accumulation in the ECM

Fibronectin is known to interact with several proteins. Some of these (e.g., integrins) help to orchestrate the polymerization of fibronectin into fibrils whereas others (e.g., fibrillin, latent TGF-β binding protein-1) require fibronectin in order to be assembled or deposited into the matrix (Sabatier, Chen et al. 2009, Dallas, Sivakumar et al. 2005). Experiments using a peptide to block fibronectin assembly in ATDC5 cells show that extra-cellular FLRT2 staining is dependent on new fibronectin fibril formation, suggesting that FLRT2 is either being re-internalized or shed into the culture medium when this process is halted.

Whereas fibronectin fibril formation was completely blocked after two days of culture with the N-terminal peptide, there appears to be a rebound in fibronectin fibril formation when cultures are grown in the same conditions for 7 days. Comparison with the 7 day control cultures demonstrates that the fibronectin matrix appears less developed, with a lesser degree of cross-linking and more solitary fibers. Fibronectin assembly is an ongoing process with continuous polymerization being required in order for the matrix to be stabilized at the cell surface – when fibronectin fibril formation subsides, the existing matrix subsides (Sottile, Hocking 2002, Wierzbicka-Patynowski, Mao et al. 2007). Cells that were treated with the inhibitor likely formed some fibronectin matrix before the blocking peptide was replenished after 4 days of culture, as seen in similar experiments using fetal rat calvarial osteoblasts (Dallas, Sivakumar et
al. 2005). Because continual fibril formation is required to stabilize the fibronectin matrix, replenishment of the N-terminal 70kDa fragment of fibronectin in the culture media would have led to the regression of the existing matrix, with a new fibronectin matrix re-emerging around day 6 or 7. Therefore, any fibronectin fibrils in these cultures are likely relatively new. In this case, FLRT2 is almost exclusively colocalized with fibronectin, mirroring previous immunolocalization experiments where cells were stained after 1 or 2 days of culture. Cells grown with vehicle only for 7 days show two striking differences in the pattern of FLRT2 staining as compared to cells cultured in the presence of the 70kDa N-terminal fragment of fibronectin: 1) FLRT2 staining is remarkably widespread with a much stronger signal, and 2) FLRT2 exhibits decreased colocalization with fibronectin. The marked decrease in FLRT2 signal in cells where fibronectin fibril formation was inhibited for 7 days corresponds to what is seen after 2 days – fibronectin fibril formation appears to be required in order for FLRT2 accumulation to occur. This relationship between FLRT2 staining and fibronectin fibril formation may indicate that FLRT2 is either being deposited directly with fibronectin, or that it may be deposited along with other fibronectin-dependent proteins. Collagen and fibrillin-1, for example, require fibronectin fibrils in order to be efficiently assembled into the ECM (Mecham 2001, Singh, Carraher et al. 2010). If FLRT2 is deposited in conjunction with either collagen or microfibrils, a decrease in fibronectin fibril formation would result in a decrease in FLRT2 staining. The decreased colocalization of fibronectin and FLRT2 in the vehicle-only cultures after 7 days implies that, as the fibronectin matrix reorganizes, FLRT2 remains where it was originally deposited in the ECM and may be interacting with other molecules here.

More information is required in order to completely elucidate the relationship between fibronectin fibril formation and FLRT2 accumulation. First, it is necessary to determine whether FLRT2 is being re-internalized or whether the protein is being cleaved from the cell surface
when fibronectin fibril formation is inhibited. If a direct interaction between FLRT2 and fibronectin exists, and if this is mediated by the N-terminal region of fibronectin, the cleaved FLRT2 ECD may be sequestered by the excess N-terminal fibronectin peptide, accounting for the observed decrease in FLRT2 signal when the blocking peptide was added to the cultures. Various experiments can be conducted in order to sort out which is the case. To further examine the possibility of FLRT2 sequestration by the fibronectin fragment, an alternate method of blocking fibronectin fibril formation, such as siRNA knockdown of fibronectin expression, could be used. A decrease in FLRT2 staining in cells where endogenous fibronectin is knocked down would confirm that this effect is specific to the process of fibronectin fibril formation and not due to binding and sequestration by the N-terminal fibronectin fragment. In this case, rescue of cells with the addition of exogenous fibronectin would re-establish the normal pattern of fibronectin fibril formation and associated FLRT2 staining. There is, of course, a possibility that extracellular FLRT2 does not accumulate in the absence of fibronectin fibril formation because the protein is being re-internalized. This can be determined by examining the relative levels of shed FLRT2 or the amount of internalized FLRT2 in cells where fibronectin fibril formation or fibronectin expression has been blocked. The former may be checked by monitoring levels of shed FLRT2 in cell-conditioned media when ATDC5 cells are grown in the absence/presence of the 70kDa N-terminal fibronectin fragment or fibronectin siRNA. Re-internalization of FLRT2 in the absence of fibronectin fibril formation would lead to a decrease in the amount of cleaved FLRT2 ECD in cell-conditioned media. The latter may be tested by subjecting ATDC5 cells to trypsinization and running cell lysates on western blots to detect FLRT2. Trypsinization of FLRT2 present at the cell membrane would result in cleavage and thus a lower molecular weight band, while intracellular/re-internalized FLRT2 would remain resistant to the enzyme and maintain its original molecular weight. If inhibition of fibronectin fibril formation does in fact
lead to increased FLRT2 internalization, cells cultured in the presence of the 70kDa N-terminal fragment of FLRT2 would be expected to show a higher level of trypsin-resistant FLRT2 as compared to untreated cells. Together, these experiments might help to shed some light on the functional interaction between FLRT2 and fibronectin.

4.6 Hypothetical Location and Functions of FLRT2 in ATDC5 Cells

FLRT2 proteins have traditionally been characterized as transmembrane proteins, with research focusing mainly on their roles in FGF signaling and regulating adhesion at the cell surface. More recently, however, the discovery of shed, functional FLRT ECDs that are capable of exerting effects non-cell autonomously has challenged that paradigm (Yamagishi, Hampel et al. 2011). The present body of work provides further evidence supporting the idea that, in addition to their cell-autonomous functions, the FLRT proteins are shed from the cell surface and may thereby affect the surrounding environment.

Based on the results of this study, a hypothesis with respect to the location and function of FLRT2 in the pre-chondrogenic ATDC5 cell line can be formulated. These findings suggest that FLRT2 is processed as a transmembrane protein and is present at the membrane for a (likely brief) period of time. Here, it may contribute to early cell-ECM interactions and may also be responsible for the transduction of signals from the external environment to the inside of the cell via its cytoplasmic tail, possibly in coordination with other cell surface receptors, ECM molecules, and intracellular adaptor proteins. The putative interaction between fibronectin and FLRT2 implies that FLRT2 may be cooperating either directly with fibronectin or with other cell-surface and intracellular proteins in areas where cells interact with fibronectin. FLRT2 is
then cleaved and deposited into the ECM where it may function as a growth factor, a scaffold for other proteins, or even as a chemoattractant/chemorepellent to surrounding cells.

This hypothesis, though preliminary, provides a working model with which to formulate further experiments. Once the cleavage of FLRT2 has definitively been proven/disproven and its interaction with fibronectin has been further characterized, studies can explore the involvement of FLRT2 in mediating cell-ECM interactions as well as its potential non-cell autonomous functions.

4.7 The Big Picture

The embryonic expression pattern of the FLRT proteins indicates that they are important players in developmental processes. Further to this, functional studies have demonstrated that FLRT2 and FLRT3 are necessary for normal heart development and that they are involved in guiding neuronal migration. This data provides evidence that FLRT2 may be involved in mediating cell-ECM interactions, either cell-autonomously and/or non-cell autonomously. It is interesting to consider this in the context of neural crest cell migration and pre-chondrogenic cellular condensation, since the developmental expression pattern of FLRT2 has suggested that it may be involved in both these events (Gong, Mai et al. 2009). That FLRT2 has been shown to interact with the ECM, likely in cooperation with fibronectin, fits well with these putative roles.

Cellular migration is an elaborate process that involves the coordination of cell polarization, adhesion and actin polymerization at the leading edge of cells, and de-adhesion and actin de-polymerization at the trailing edge of cells (Ridley, Schwartz et al. 2003). The ECM can contribute to this in two important ways. First, it may be the source of soluble factors that act as migration signals (i.e. chemoattractants or chemorepellents) to the surrounding cells. The findings of Yamagishi et al. indicated that the ectodomain of FLRT2 plays a role in guiding
neuronal migration in cooperation with the UNC5d cell-surface receptor (Yamagishi, Hampel et al. 2011). In this case, shed FLRT2 acts as a chemorepellent to UNC5d-positive neurons, delaying their movement from the subventricular zone to the cortical plate. The second way in which the ECM contributes to cell migration is through the formation of cell-ECM adhesions that are crucial in stabilizing cell protrusions, thus providing the traction force necessary for cellular propulsion (Ridley, Schwartz et al. 2003). Integrins are the major players in forming these cell-ECM adhesions, and the fibronectin receptor (integrin α5β1) has been shown to be an important component of both 2-dimensional fibrillar adhesions and 3-dimensional matrix adhesions (Berrier, Yamada 2007, Cukierman, Pankov et al. 2002, Cukierman, Pankov et al. 2001). It therefore follows that cellular interactions with fibronectin are crucial to cellular migration. Indeed, previous research indicates cellular interactions with fibronectin are important in CNCC migration (Rovasio, Delouvee et al. 1983, Bronner-Fraser 1986). It is well established that cell-ECM interactions are essential to the process of pre-chondrogenic cell condensation (Hall, Miyake 2000). Fibronectin is probably one of the most important ECM molecules in this respect, as it has been shown to play a critical role in cell condensation both in vitro and in vivo (Georges-Labouesse, George et al. 1996, Frenz, Jaikaria et al. 1989, Gehris, Stringa et al. 1997, White, Hershey et al. 2003). Thus, both CNCC migration and cellular condensation are dependent on the interaction of cells with the ECM, and in particular fibronectin. The idea that FLRT2 could be mediating cell-ECM interactions, and that it may be cooperating with fibronectin in doing so, provides a link between the functional inferences based on expression data and the current experimental results.
Chapter 5 – Limitations

5 Limitations of the Study Design

The main limitation of this study relates to the use of an *in vitro* model that employs a transformed cell line. As with any *in vitro* system, there is always a concern that what is being observed does not necessarily reflect *in vivo* state. This is particularly true when studying cell-ECM adhesions since we know that cells behave differently in 2-D versus 3-D systems (Berrier, Yamada 2007, Cukierman, Pankov et al. 2001, Cukierman, Pankov et al. 2002). Not only do cell-ECM adhesions in 3-D systems have a composition that is distinct from those found in 2-D cultures, but cell shape and migration are altered as well. Future experiments may include the use of whole tissues preparations, primary cells, or cell-derived 3D matrices in order to simulate *in vivo* conditions more closely.

Expression studies have implicated FLRT2 in important developmental processes such as CNCC migration and pre-chondrogenic cell condensation. These do not take place in environments that are homogenous but instead occur in the context of growth factor gradients and populations of cells committing to distinct differentiation pathways. The use of a relatively homogenous cell line therefore runs the risk of missing any effects that FLRT2 may have on other cell types. This is of particular concern when considering the potential non-cell autonomous functions of the protein. Yamagishi *et al* (2011) demonstrated that FLRT2 may exert its effects in a region of the brain that is relatively distant from where it is being expressed. Therefore, it is possible that important effects of FLRT2 on cell populations that are completely distinct from pre-chondrocytes will be missed in the ATDC5 cell model.
Chapter 6 – Future Directions

6 Future Directions

In order to further develop our knowledge of the role that FLRT2 plays during embryological development, future experiments may be focused on one of the following:

1) Elucidating how FLRT2 is functioning in relation to cell-ECM adhesions

2) Investigating the nature and functional aspects of the putative interaction of cleaved FLRT2 with the ECM

The first goal might involve studying FLRT2 in the context of specific types of cell-ECM adhesions such as focal adhesions, fibrillar adhesions, or 3-D matrix adhesions. This could involve further immunolocalization experiments to look at the distribution of FLRT2 relative to the other proteins that are found in these areas (e.g. tensin and integrin α5β1, in the case of fibrillar adhesions). The physical interaction between FLRT2 and these proteins can also be investigated in vitro using a range of methods such as co-immunoprecipitation, yeast two hybrid assays, and BRET/FRET assays. If physical interactions are indicated, functional interactions can then be studied. The integrin “adhesome” (cell surface and intracellular proteins that are involved in integrin-mediated cell adhesion) is very complex and has been shown to contain more than 180 proteins that function at different levels (Geiger, Yamada 2011). The task of determining if and how FLRT2 is involved in the adhesome may prove to be challenging.

The second goal of determining how shed FLRT2 interacts with the ECM can be partly achieved by assessing physical interactions between FLRT2 and other ECM proteins. Because many ECM proteins are relatively large and somewhat hard to purify, this may be difficult. The relationship between FLRT2 and other fibronectin-dependent proteins in the ECM should also be investigated. Collagen and fibrilin-1 both require fibronectin in order to be assembled in the
ECM – there exists a possibility that FLRT2 is interacting with these molecules as well.

Interestingly, the small leucine rich proteoglycans that closely resemble FLRT2 in structure are known to be involved in the orientation of collagen fibers – perhaps secreted FLRT2 might be involved in a similar manner (Mecham 2001). Alternately, the non cell-autonomous function of cleaved FLRT2 may be investigated by testing the effects of the addition of exogenous FLRT2 ECD to cell cultures. Conversely, FLRT2 expression can be knocked down using antisense RNA technology. It would be interesting to see whether the levels of FLRT2 ECD present in cultures affects either: a) the formation of other ECM molecules such as collagen or fibrillin-1, 2) cell migration characteristics, and 3) the formation of cell condensations and subsequent chondrogenic differentiation. The shed form of FLRT2 has already been implicated in cell migration; however, it has not yet been studied specifically in relation to cell condensation and chondrogenesis.
7 Conclusions

In this study, I demonstrated the sub-cellular localization of FLRT2 in the pre-chondrogenic ATDC5 cell line. FLRT2 is found at the cell membrane, but also appears to be in the ECM. Further to this, the evidence suggests that FLRT2 may be involved in mediating cell-ECM interactions.

Additional conclusions can be drawn from the data, with the caveat that further experiments are required in order to solidify the supporting evidence:

1) FLRT2 is cleaved from the surface of ATDC5 cells, with the ECD accumulating in the ECM
2) FLRT2 interacts with fibronectin, either directly or in the same complex of cellular proteins found at cell-ECM adhesions to fibronectin
3) FLRT2 accumulation in the matrix is dependent on fibronectin fibril formation

These results open up many possibilities as to the functions of FLRT2 during embryological development. In addition to its previously proposed roles in regulating signaling and cell adhesion at the cell surface, shed FLRT2 may possess additional functions in the ECM. These could involve regulating cellular migration, coordinating the formation of matrix fibers, providing structural support in coordination with other matrix fibers, or potentially acting as a soluble growth factor. This work provides a platform for future research that has the promise to be both exciting and challenging.
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