DEVELOPMENTAL EXPRESSION PATTERN OF JUNCTIONAL ADHESION MOLECULE-A (JAM-A)

By

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ABSTRACT

Cell adhesion molecules of the Ig superfamily play an important role in embryonic development. It has recently been shown that JAM-A, a member of this family expressed at endothelial and epithelial tight junctions, is involved in platelet activation, leukocyte transmigration, and angiogenesis. Here I determine the expression pattern of the JAM-A gene during embryogenesis using transgenic mice expressing lacZ under the control of the endogenous JAM-A promoter. Histochemical staining for β-galactosidase in heterozygous mouse embryos was first seen in the inner cell mass and trophectoderm of the blastocyst. By 8.5 dpc, JAM-A gene activity was detected in the endoderm and part of the surface ectoderm. At 9.5 dpc, JAM-A expression began to localize to certain organ systems, most notably the developing inner ear and early vasculature. Localization of JAM-A to embryonic vasculature was confirmed by double-staining with antibodies against JAM-A and PECAM-1, a known endothelial cell marker. As organogenesis progressed, high levels of JAM-A expression continued in the epithelial component of the inner ear as well as the epithelium of the developing skin, olfactory system, lungs, and kidneys. In addition, JAM-A gene activity was found in the developing liver, choroid plexuses, and gut tubes. Immunofluorescent staining with a JAM-A antibody was performed to confirm that expression of the JAM-A-β-galactosidase fusion protein accurately represented endogenous JAM-A protein. Thus, JAM-A is prominently expressed in embryonic vasculature and the epithelial components of several organ systems.
Chapter 1

INTRODUCTION

Junctional adhesion molecule-A (JAM-A or JAM-1) is a 32-kDa type-1 transmembrane glycoprotein in the immunoglobin (Ig) superfamily of molecules. JAM-A contains two extracellular domains, a single transmembrane domain, and a short cytoplasmic tail (Figure 1, Bazzoni, 2003; Naik and Eckfeld, 2003).

![Figure 1](image_url)

Figure 1  A schematic of the JAM-A protein showing the two extracellular domains above the membrane and the short cytoplasmic tail. Taken from Naik and Eckfeld, 2003.

The furthest N-terminal extracellular domain is a VH-type Ig like domain while the membrane-proximal extracellular domain is a C2-type Ig domain. The transmembrane domain contains 17 hydrophobic amino acids attached to the 45 amino acid
cytoplasmic domain, which facilitates several intracellular interactions (Naik and Eckfeld, 2003). JAM-A is found at the tight junctions of epithelial and vascular endothelial cells and interacts with tight junction-associated proteins such as zona occludens-1 (ZO-1), AF-6, and partitioning-defective (PAR) protein 3 through a PDZ-binding domain (Ebnet et al., 2000; Ebnet et al., 2001). It is thought that through these interactions JAM-A may help recruit other proteins during the formation of tight junctions. JAM-A is also known to interact with other JAM-A molecules on adjacent cells at the tight junction and with integrins LFA-1 and $\alpha_v\beta_3$ (Bazzoni, 2003). The homophilic interactions of JAM-A occur at the N-terminus of the extracellular domain and involve the formation of a salt bridge between adjacent R(V,I,L)E dimerization motifs (Kostrewa et al., 2001). Because JAM-A is only observed at tight junctions in which it is expressed in two neighboring cells, the homophilic interaction is thought to be fundamentally important to the role of JAM-A in tight junction regulation (Naik et al., 2001).

JAM-A was first cloned as a receptor for the platelet monoclonal antibody F11 (M.Ab.F11). It was found to be involved in platelet adhesion to Chinese Hamster Ovary (CHO) cells (Naik et al., 2001). Additional studies have shown that JAM-A promotes platelet-endothelial cell adhesion and that soluble JAM-A inhibits platelet aggregation, adhesion, and potentiation induced by M.Ab.F11 (Babinska et al., 2002a and Babinska et al., 2002b). JAM-A is also known to be involved in leukocyte transmigration through direct interactions with the integrin LFA-1, suggesting that it may be important in the immune response (Ostermann et al., 2002). Finally, it has been demonstrated that JAM-A is a receptor for reovirus, involving an interaction with the viral attachment protein, $\sigma_1$. Treating cells with anti-JAM-A antibodies inhibits
viral binding and prevents reovirus infection. In addition, cells lacking endogenous JAM-A expression are found to be resistant to reovirus infections, while transiently transfecting these cells with JAM-A permits viral infection (Barton et al., 2001).

The Naik laboratory has recently shown that blocking JAM-A activity using small interfering RNA (siRNA) prevents basic fibroblast growth factor- (bFGF) induced endothelial cell migration (Naik et al., 2003b). Further, it was shown that the migration of endothelial cells on vitronectin is promoted by JAM-A in an αvβ3-dependent manner, and, somewhat surprisingly, overexpressing JAM-A in endothelial cells does not increase cell adhesion, but rather promotes cell migration (Naik and Naik, 2006). An important physiological process requiring endothelial cell migration is angiogenesis. Angiogenesis is the process of forming new blood vessels by branching off pre-existing ones. The Naik laboratory found that JAM-A is important for bFGF-induced angiogenesis. A chick chorioallantoic membrane assay shows that treatment with bFGF is capable of stimulating angiogenesis; however, by first treating the blood vessel with an inhibitory antibody against JAM-A, bFGF is no longer capable of inducing angiogenesis (Figure 2, Naik et al., 2003a).
A chick chorioallantoic membrane assay shows the importance of JAM-A in angiogenesis. A blood vessel (upper left) will undergo angiogenesis when treated with bFGF (upper right). However, when treated with bFGF and an inhibitory antibody against JAM-A, angiogenesis is reduced (lower right). Modified from Naik et al., 2003a)

These studies have been significantly expanded by studying the phenotype of JAM-A null mice. While the mice are viable and appear to live as long as wild type mice, they show signs of cardiovascular defects that provide interesting insights into the function of JAM-A. Despite any apparent change in the general vasculature, the mice have been found to have enlarged hearts due to dilated cardiomyopathy. The mice also show a decrease in bFGF-induced angiogenesis that mimics the Naik laboratory’s findings in cell culture studies. This has been established using both aortic ring assays and matrigel assays from the JAM-A null mice designed to study blood vessel formation. In addition, it was found that the mice up-regulate vascular endothelial growth factor (VEGF), another growth factor known to stimulate
angiogenesis but not in a JAM-A-dependent manner. This increase in expression is accompanied by an up-regulation of one of the VEGF receptors, VEGFR2, and an increase in VEGF-induced angiogenesis (Cooke et al., unpublished data). These data indicate that although mice lacking JAM-A show signs of cardiovascular problems, there is some degree of compensation that occurs in order to allow angiogenesis.

The role of JAM-A in angiogenesis suggested that it may also have a role in vasculogenesis, the initial formation of blood vessels during embryonic development. Vasculogenesis in the mouse begins around 7.5 days post coitum (dpc) when the visceral endoderm signals the mesoderm to differentiate into endothelial cells and erythroblasts (Bohnsack and Hirschi, 2004). This differentiation involves a developmentally common transition from epithelial cells to mesenchyme. In this process, angioblasts are induced from the mesoderm and then begin to migrate and adhere into blood islands, shaping an early vascular pattern (Poole et al., 2001). As vasculogenesis continues, the endothelial cells form through differentiation of the angioblasts, replicate, and adhere to build a primitive capillary plexus, or early blood vessel, around 8.5 dpc (Figure 3).
Figure 3 A general diagram showing the steps of vasculogenesis. First, the visceral endoderm signals the mesoderm prompting an epithelial to mesenchymal transition. Angioblasts are then induced from the mesoderm to form blood islands, which develop into primitive blood vessels composed of endothelial cells.

Mature blood vessels form after a process involving endothelial cell maturation and migration, followed by the recruitment of smooth muscle cells. One of the important growth factors in this process is bFGF (Bohnsack and Hirshi, 2004), which is known to depend on JAM-A activity during angiogenesis. Because of this relationship, we were interested in exploring where JAM-A was expressed during embryonic development, specifically if it was found at sites of vasculogenesis. It was also known that other developmental processes, such as the reorganization of epithelia during morphogenesis and organogenesis, require control of tight junction formation, cell adhesion, and cell migration (Denker and Nigam, 1998; Mlodzik, 2002). Thus, we believed that JAM-A may have an important role in numerous processes during embryogenesis.

Northern blot analysis of adult human tissues showed that JAM-A was localized to the heart, placenta, lung, liver, kidney, and pancreas (Naik et al., 2001).
Previous reports have shown that JAM-A mRNA and protein is found in preimplantation human and mouse embryos and in several epithelial tissues (Ghassemifar et al., 2003; Martin-Padura et al., 1998). However, JAM-A mRNA was shown to be absent from mouse embryos ranging from 7 to 17 dpc using northern blot analysis (Aurrand-Lions et al., 2001). In order to conduct a detailed study of JAM-A expression during embryonic development, I used transgenic mice containing a lacZ-tagged gene trap insertion in the JAM-A gene as well as immunofluorescence staining for the JAM-A protein. I found that JAM-A expression begins early in development and becomes localized to various organ systems as development progresses.
Chapter 2
MATERIALS AND METHODS

2.1 The generation of transgenic mice

Transgenic mice have become an important and widely used tool for studying the function and expression of a variety of genes in vivo. The first stage of generating transgenic mice involves identifying a gene of interest and designing a construct to interrupt it. In order to target a specific gene, a construct can contain flanking regions which are homologous to the gene of interest. These will facilitate a recombination event inserting the construct into the desired gene. The construct also contains genes for one or more enzymes allowing histological detection of cells or tissues expressing the gene in which it has been inserted. Common enzymes include the E. coli β-galactosidase enzyme encoded by the lacZ gene or the placental alkaline phosphatase enzyme. Finally, the construct will encode an antibiotics resistance gene allowing transfected cells to grow in the presence of chemicals such as neomycin or hygromycin.

Once a construct has been designed it can be transfected into mouse embryonic stem (ES) cells by electroporation. In a small percentage of the cells it will be incorporated into a chromosome under the control of an active gene’s promoter and will be expressed. Cells expressing the construct can be selected by treatment with antibiotics. The genomic location of the construct can be determined by restriction enzyme digestion and Southern blotting or PCR, in the case of targeted integration.
into a gene, or by 5’ rapid amplification of cDNA ends (5’-RACE) for random insertions.

Mouse ES cells expressing the construct can then be microinjected into a blastocyst. The blastocyst is then injected into a pseudo-pregnant female, which will eventually give birth to a chimeric mouse containing some cells and tissues heterozygous for the allele containing the construct. These chimeras can then be bred to produce fully heterozygous offspring.

The mice used in my work were obtained from a collaboration with Dr. William Skarnes. The mice were originally found as part of a large study designed to specifically target secreted and transmembrane proteins. The construct, or “gene trap” vector, was found to have randomly inserted into the JAM-A gene (Mitchell et al., 2001). Its location was determined using the 5’-RACE technique and upon request we were sent mice heterozygous for the JAM-A-gene trap allele.

2.2 Animals

All experiments using animals were approved by the University of Delaware Institutional Animal Care Committee. Heterozygous mice harboring the JAM-A β-geo-tagged allele were generated from the KST235 embryonic stem (ES) cell line created by an insertion of the pGT1pf5 gene trap vector (Mitchell et al., 2001). The KST235 sequence tag and ES cell line are available from BayGenomics (http://baygenomics.ucsf.edu). Embryonic mice were staged by designating noon of the day on which a semen plug was observed in the dam as 0.5 days post-coitum (dpc). When a pregnancy had proceeded to the desired stage, the mice were sacrificed by cervical dislocation and embryos were dissected. All mice were maintained in a 12-hour light/dark cycle at 21-24°C and were given food and water ad libitum.
2.3 Histochemical staining of pre-implantation mouse embryos for β-Galactosidase activity

Wildtype female mice were injected with 5 IU of pregnant mare serum gonadotropin (National Hormone and Peptide Program, Torrance, California, USA) followed 46 hours later by 5 IU of human chorionic gonadotropin (Sigma-Aldrich Co. St. Louis, Missouri, USA) then mated with males homozygous for the JAM-A gene trap allele. Seventy two hours later, embryos at the 8-cell to early morula stage were collected and cultured in vitro for 24-48 hours at 37°C and 5% CO₂ in a sterile 60 mm tissue culture dish containing 10 μL microdrops of media that were overlayed with light paraffin (mineral) oil. The media used was KSOM with ½ amino acids, glucose, and phenol red (Specialty Media cat. no. MR-121-D). The microdrops were overlayed with embryo-tested mineral oil (Sigma-Aldrich cat. no. M-8410).

Pre-implantation embryos were washed three times for seven minutes each at RT in phosphate-buffered saline (PBS) for tissue culture (Invitrogen). Embryos were fixed for 30 minutes at 4°C in PBS containing 1% paraformaldehyde, 0.02% glutaraldehyde, and 5mM EGTA and washed again in PBS three times for seven minutes each. The embryos were transferred to staining solution (PBS containing 0.1% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆, 2mM MgCl₂, 0.02% Nonidet P-40, and 5mM EGTA) and incubated at 30°C in the dark for the times noted in the text. Pre-implantation embryos were then washed three times for seven minutes each at room temperature in 3% dimethylformamide in PBS before being photographed on a light microscope.
2.4 Histochemical staining of postimplantation embryos for β-Galactosidase activity

Embryos from 8.5 until 11.5 dpc were isolated and whole embryos incubated at 4°C for one hour in fixation solution (PBS containing 1% paraformaldehyde, 0.2% glutaraldehyde, 0.02% NP-40, and 0.01% sodium deoxycholate). Specimens were then washed three times (7-10 minutes each) in PBS, and stained for β-galactosidase activity at 30°C overnight in an X-gal solution (50 mM K₃Fe(CN)₆, 50 mM K₄Fe(CN)₆, 0.02% NP-40, 0.01% sodium deoxycholate, 2 mM MgCl₂, and 1 mg/ml X-gal (from 40 mg/ml stock in dimethyl formamide) in PBS). Specimens were then washed three times (7-10 minutes each) with PBS containing 3% DMF and then post-fixed in 4% paraformaldehyde in PBS for 30 min at 4°C. Finally, specimens were rinsed once in PBS with 3% DMF and stored in the dark at 4°C in the same solution. Embryos in these steps were kept in a 24-well cell culture dish (Corning) that allowed them to be manipulated easily and minimized the amount of fixation and staining solutions needed. Staining was visualized either by clearing the embryos as described below or by embedding them in OCT (Tissue Tek), sectioning at 20 μm on a cryostat, mounting the sections on slides and coverslipping with Gel mount (Biomedia, Foster City, California, USA) mounting media.

Methyl salicylate was used to clear X-gal stained whole embryos for photodocumentation as described at (http://www.paperglyphs.com/wmc/docs/lacZ_bible.html). Briefly, embryos were washed with distilled water twice for 30 minutes at room temperature and subsequently dehydrated with agitation at room temperature as follows: 30 minutes in 70% ethanol, 30 minutes in 95% ethanol, twice in 100% ethanol for 30 minutes each. Embryos were then transferred to 100% methyl salicylate and agitated at room
temperature until the tissues cleared (approximately 15 minutes). Cleared embryos were photographed through a dissecting microscope within one hour of clearing to prevent loss of the blue color resulting from X-gal staining.

Embryos at 12.5 and 13.5 dpc were dissected, embedded immediately in OCT, sectioned at 20 μm on a cryostat, incubated at 4°C for one hour in fixation solution (PBS containing 1% paraformaldehyde, 0.2% glutaraldehyde, 0.02% NP-40, and 0.01% sodium deoxycholate), stained for β-galactosidase activity and coverslipped as above. For embryo sections on microscope slides, the fixation and rinsing steps were done in Coplin jars, while the X-gal solution was applied directly to the slides while laying flat in a humid chamber.

2.5 Enzyme immunohistochemistry

Enzyme immunohistochemical staining for PECAM-1 was performed on X-gal stained sections as described (Wakayama et al., 2003). Briefly, sections generated from whole mount X-gal staining of 11.5 dpc embryos as described above were treated with Triton X-100 in PBS for one hour, washed with PBS, and then immersed in 3% H2O2 in methanol for ten minutes. Sections were then incubated with 3% BSA in PBS for 30 minutes and then incubated overnight at 4°C with a PECAM antibody (rat anti-mouse PECAM-1/CD31 antibody, catalog number 550274, BD-Pharmingen) diluted to 1 μg/ml in PBS. This was followed by incubation with biotinylated anti-rat IgG antibody for one hour at RT. Sections were then treated with horseradish peroxidase-labeled streptavidin for one hour at RT and incubated with 3′3′-diaminobenzidin tetrahydrochloride (DAB) until a light brown color was observed. All solutions were applied directly to slides in a humid chamber with the
exception of the treatment with 3% H$_2$O$_2$ in methanol, which was done in a Coplin jar. Any steps using the DAB reagent were done in a fume hood.

### 2.6 Fluorescent immunohistochemistry

The immunofluorescence procedures were taken from Reed et al. (Reed et al. 2001). Briefly, unfixed cryosections were fixed at -20°C for 20 minutes in 1:1 acetone:methanol. The sections were then blocked in 1% BSA in PBS at 4°C for one hour followed by incubation with goat polyclonal anti-mouse JAM-A antibody (1:100 dilution, catalog number AF1077, R&D systems) at room temperature for one hour. Sections were then washed 2-3 times in 1X PBS for 5-10 minutes each and incubated in the dark for one hour at room temperature with AlexaFluor 568 donkey anti-goat IgG antibody (1:50 dilution, Molecular Probes, Eugene, OR) and the nuclear counterstain TO-PRO-3 (1:3000 dilution, Molecular Probes). Double labeling for JAM-A and PECAM-1 expression was performed similarly, except that rat anti-mouse PECAM-1/CD31 antibody (1:15.6 dilution, BD-Pharmingen) was added to the primary antibody solution and AlexaFluor 488 conjugated anti-rat IgG (Molecular Probes) was added to the secondary antibody solution. Blocking and antibody incubation steps were performed in a humid chamber as described above. Slides were stored at -20°C until photographed on a Zeiss LSM 510 Confocal microscope configured with an Argon/Krypton laser (488 nm and 568 nm excitation lines) and Helium laser (633 nm excitation line)(Carl Zeiss Inc., Göttingen, Germany).
3.1 JAM-A Gene Trap Insertion

A DNA construct was inserted into the JAM-A gene between the fourth and the fifth exons in mouse embryonic stem cells. The gene trap construct contains a CD4 transmembrane domain attached to the coding regions of the active sites for β-galactosidase and neomycin transferase, and the placental alkaline phosphatase gene separated by an internal ribosome entry site (Skarnes et al., 1995). The result of this is the expression of a fusion protein under the control of the endogenous JAM-A promoter containing the first extracellular domain of JAM-A attached to the β-galactosidase and neomycin transferase active sites (Figure 4).
Figure 4  The JAM-A gene (upper left) is shown with the gene trap construct inserted between the fourth and fifth exons (lower left), and the resulting chimeric mRNA (upper right) and proteins (lower right). Abbreviations: β-geo, β-galactosidase-neomycin fusion gene/protein; IRES, internal ribosome entry site; PLAP, placental alkaline phosphatase; TM, transmembrane domain.

Heterozygous mice created from these stem cells were used to study JAM-A gene activity by assaying for β-galactosidase activity with the chemical X-gal, which is cleaved by β-galactosidase to form a blue precipitate (Figure 5; Cepko et al., undated). Embryos treated with X-gal would then turn blue in tissues where JAM-A was being expressed.
Figure 5  The β-galactosidase enzyme cleaves a galactose molecule off the chemical X-gal, resulting in a 5-bromo-4-chloro-indoxyl group. Two of these groups can dimerize under oxidative conditions to form a stable, blue precipitate. Modified from Cepko et al., undated.

3.2 Early Embryonic Expression of JAM-A

One of my goals was to determine when the JAM-A gene first became active in the developing embryo. To do this, it was necessary to stain embryos at the morula and blastocyst stage for β-galactosidase activity indicative of JAM-A gene expression. JAM-A expression is first seen as the morula transitions to the blastocyst (Figure 6).
JAM-A expression begins as the morula (A) transitions to the blastocyst (B). Abbreviations: bc, blastocoele; icm, inner cell mass; tr, trophectoderm; zp, zona pellucida. Blue/green color indicates JAM-A gene activity as detected by β-galactosidase staining.

While no gene activity was detected in the morula after 7 hours of staining, weak staining was observed after 20 hours (data not shown). JAM-A expression was found in the blastocyst after 7 hours of staining in the inner cell mass and the trophectoderm. This expression pattern is supported by previous detection of JAM-A protein in mouse and human preimplantation embryos (Thomas et al., 2004).

By 8.5 dpc, JAM-A expression is seen broadly throughout the ventral side of the embryo (Figure 7A) as well as in the chorion (data not shown). After sectioning it was evident that JAM-A was present in the endoderm, surface ectoderm, and foregut diverticulum. However, little JAM-A gene activity was observed in the mesoderm, neural plate/fold, heart, or allantois (Figure 7B-D). The absence of significant levels of JAM-A in the heart was a notable trend throughout embryonic development. Staining of adult hearts showed that JAM-A was expressed only in the blood vessels of the heart, but not in the majority of heart tissue (data not shown).
Figure 7  JAM-A is broadly expressed in the ventral region of the 8.5 dpc wholemount embryo (A). Sectioning (B-D), denoted by black lines in A, shows that expression is specifically localized to the surface ectoderm, foregut diverticulum, and endoderm. Abbreviations: al, allantois; ed, endoderm; et, surface ectoderm; fd, foregut diverticulum; hrt, heart; ht, heart tube; md, mesoderm; ne, neuroepithelium; nt, neural tube. Blue/green color indicates JAM-A gene activity.

At 9.5 dpc, expression remains prominent in the ventral portion of the embryo and is beginning to localize to various organ structures as they begin to develop (Figure 8A). Differentiation generally occurs from the rostral to caudal regions of the embryo, as is observed in somite formation. JAM-A is seen to follow this pattern, as the rostral region of the 9.5 dpc embryo expresses less JAM-A than the caudal region. By 12.5
dpc, JAM-A expression is highly tissue-specific, while wild-type embryos at the same age, with no gene-trap, do not stain for β-galactosidase activity (Figure 8B and C).

![Image of embryos showing expression of JAM-A](image)

**Figure 8** Wholemount expression of JAM-A is detected by X-gal staining in 9.5 dpc (A) and 12.5 dpc heterozygous (B) embryos, but not in a 12.5 dpc wild-type (C) embryo. Abbreviations: gt, gut tube; ie, inner ear; isv, intersomitic vessels; op, otic pit/vesicle. Blue/green color indicates JAM-A gene activity.

### 3.3 JAM-A Expression in Embryonic Blood Vessels

Blood vessels first appear in the developing embryo through the process of vasculogenesis. Blood islands are formed from the clustering of mesoderm cells and consist of a core of hemopoietic cells surrounded by angioblasts. Blood islands fuse together as the angioblasts differentiate into the endothelial cells that line the lumen of a blood vessel (Risau and Flamme, 1995). This process is known to occur in the allantois, where angioblast- and blood vessel-specific proteins such as Tal1/Flk1 and PECAM-1, respectively, are present (Drake and Fleming, 2000). However, in the 8.5 dpc embryo, JAM-A is absent from the allantois, suggesting that it is not involved
in the early processes of vasculogenesis. The potential role of JAM-A during blood vessel development is discussed in greater detail below.

While JAM-A was not observed in the allantois (Figure 7A), it is present in blood vessels of 9.5-12.5 dpc embryos as determined by X-gal staining (Figures 8A,B and 9A-C). At 9.5 dpc, staining was seen at sites of intersomitic vasculature and in cranial vasculature of 11.5 and 12.5 dpc embryos. To confirm that JAM-A was localized to blood vessels, sections were stained for a known blood vessel marker, PECAM-1 (Albelda et al. 1991; Ilan and Madri, 2003). JAM-A and PECAM-1 were shown to colocalize through both histochemical (Figure 9A-C) and immunofluorescent (Figure 9D-F) staining of cranial sections of 11.5 dpc embryos.
Figure 9  JAM-A is expressed in the developing vasculature as indicated by β-galactosidase staining (A-C) and immunostaining (D-F). Cranial blood vessels can be seen in a wholemount 12.5 embryo head (A). Colocalization of JAM-A (blue) and PECAM-1 (brown) is seen in the head of an 11.5 dpc embryo (B) and with a higher magnification (C). Immunostaining of an 11.5 dpc embryo section also shows colocalization of JAM-A (red, D) and PECAM-1 (green, E) with the merged image (yellow, F). Abbreviations: cv, cranial vasculature; fv, fourth ventricle; msv, mesencephalic vesicle; oe, olfactory epithelium; tcv, telencephalic vesicle. A-C: Blue/green indicates JAM-A gene activity; brown indicates PECAM-1 activity. D-F: Red indicates JAM-A protein; green indicates PECAM-1 protein; yellow is merged; and blue shows TO-PRO-3 nuclear counterstain.

The X-gal staining pattern of JAM-A originally led me to believe that JAM-A was selectively marking the blood islands because, upon magnification, staining did not appear to be continuous in the blood vessel. While the PECAM-1 staining in brown showed an uninterrupted vascular structure, the blue JAM-A staining only highlighted
small circular regions within the blood vessel. To determine if this was an artifact of the staining procedure I also used fluorescent antibodies against both JAM-A and PECAM-1 proteins to see how their expression overlapped. The antibody staining suggested that my previous observation of JAM-A only in certain regions of the blood vessel was a consequence of the staining procedure. This also served to validate using the gene trap to study JAM-A gene activity by showing that antibody detection of the JAM-A protein gave the same general expression pattern as X-gal staining. Further confirmation of the X-gal staining is given below, in a number of other tissues. This characterization of JAM-A expression is consistent with previous reports that JAM-A is found in the human microvessels that form the blood-brain barrier (Vorbrodt and Dobrogowska, 2004) and the general observation that JAM-A is present in endothelial cells.

3.4 JAM-A is Expressed in the Developing Sensory Organs

One of the most notable sites of JAM-A gene expression detected by β-galactosidase staining was found in the developing inner ear (Figure 10). The inner ear develops from the head ectoderm and first appears in the embryo as the otic placode at around 8.5 dpc (Riley and Phillips, 2003). JAM-A was not seen in this structure, however as the otic placode invaginates and forms the otic pit/vesicle, the JAM-A gene becomes strongly expressed. The otic vesicle (or otocyst) is the source of other substructures of the inner ear such as the endolymphatic diverticulum, which can be seen at 11.5 dpc, and the utricle and saccule, which are formed by 12.5 dpc. JAM-A expression is found in each of these structures.
Figure 10  JAM-A is strongly expressed in the inner ear of 9.5 dpc (A), 11.5 dpc (B), and 12.5 dpc (C) embryos. Expression is also seen in the structures which form from the inner ear at 11.5 and 12.5 dpc. Abbreviations: ed; endolymphatic diverticulum; fb, forebrain; fv; fourth ventricle; mba, mandibular component of first brachial arch; op, otic pit; oph, oropharynx; ov, otic vesicle; sc, saccule; ut, utricle. Blue/green color indicates JAM-A gene activity; pink is nuclear counterstain.

The olfactory system, like the otic system, is formed through placodal development. The surface ectoderm beneath the developing forebrain becomes the nasal placode, which then forms the nasal pits and eventually the nasopharynx. JAM-A gene activity is first seen in olfactory epithelium of the nasal pits at 11.5 dpc. Expression continues as the nasopharynx forms and becomes continuous with the oropharynx and trachea at 12.5 dpc (Figure 11).
Figure 11  JAM-A expression in the developing olfactory system can be seen in the nasal pits of an 11.5 dpc wholemount embryo (A) and in the olfactory epithelium of an 11.5 dpc embryo section (B). As development progresses, JAM-A expression is continuous throughout the nasopharynx and oropharynx of the 12.5 dpc embryo (C). Abbreviations: lj, lower jaw; np, nasal pits; nph, nasopharynx; oe, olfactory epithelium; oph, oropharynx; tng, tongue. Blue/green color indicates JAM-A gene activity; pink is nuclear counterstain.

The eye, similar to the ear and olfactory system, develops from the lens placode. However, JAM-A gene activity is not found in this structure or in the developing eye through 12.5 dpc, while it has been observed in the blood vessels of the tunica vasculosa (L-I Kang and MK Dunacan; unpublished data).

3.5 JAM-A is Expressed during Branching Morphogenesis

Both the lung and the kidney form by a process known as branching morphogenesis. The lung is initially formed by branching of the pharynx and then bifurcating into the lung buds (Shannon and Hyatt, 2004). JAM-A is found in the lung buds in wholemount staining and sections of the 11.5 dpc embryo. It is also seen in the trachea, pharynx, and esophagus (Figure 12A and B). These structures are continuous with the nasopharynx and oropharynx, and JAM-A expression is found in the
epithelium throughout these regions (Figure 11C). Staining is localized to the epithelial component of the lungs and continues to be expressed in the bronchi as they develop from the primary bronchial tree at 13.5 dpc (Figure 12C).

Figure 12  In the developing lungs, JAM-A gene activity is first seen in the lung buds in both an 11.5 dpc wholemount embryo (A) and section through this structure (B). As the lung develops, JAM-A becomes localized to the bronchi (C). Abbreviations: br, bronchi; es, esophagus; lb, lung bud; ph, pharynx; tr, trachea. Blue/green color indicates JAM-A gene activity; pink is nuclear counterstain.

The kidney develops via two transient structures, the pronephros and the mesonephros. The pronephric duct becomes the mesonephros after entering the mesonephric mesenchyme and begins to branch (Dressler, 2002; Vainio and Lin, 2002). By 11.5 dpc, JAM-A expression is seen in both wholemount and embryo sections (Figure 13A and B). When the mesonephric duct grows caudally and the uterine bud is formed, the metanephric kidney begins to develop (Sakurai, 2003). JAM-A is expressed in the developing glomeruli which are formed from mesenchymal to epithelial transitions induced by the tips of the branching collecting system (Figure 13C).
Figure 13  JAM-A expression in the developing kidney begins at 11.5 dpc. At this stage, staining of the mesonephros can be seen in a wholemount embryo (A) and in a section revealing the mesonephric tubules (B). As the metanephros forms, JAM-A expression becomes very strong in the renal pelvis and glomeruli (C). Abbreviations: gl, glomeruli; lv, liver; ms, mesonephros; msd, mesonephric duct; mst, mesonephric tubules; mt, metanephros; rp, renal pelvis. Blue/green color indicates JAM-A gene activity; pink is nuclear counterstain.

3.6 JAM-A is Expressed Broadly in Developing Epithelia

Several other tissues showed JAM-A gene activity by staining with X-gal. This staining also correlated well with antibody staining for JAM-A protein, providing further confirmation of the validity of the gene trap method to determine JAM-A gene expression. These include the developing skin and hair follicles, choroids plexuses, and gut (Figure 16). JAM-A expression is seen in areas of the surface ectoderm at 8.5 dpc (Figure 7) and in the head ectoderm at 9.5 dcp (Figure 10A). This expression is continuous in the epithelium of the skin and its derivatives such as vibrissae in 13.5 dpc embryos (Figure 14A and B).

In the developing brain, the only notable sites of JAM-A expression besides the blood vessels are in the choroid plexuses, structures that form from the
neural ectoderm (Thomas and Dziadek, 1993). These structures are composed of epithelia connected by tight junctions lined by blood vessels and are responsible for the secretion of cerebro-spinal fluid (CSF) (Sturrock, 1979). JAM-A gene activity is first seen by β-galactosidase staining at 12.5 dpc in the epithelial component of the newly forming choroid plexus (data not shown). JAM-A expression continues at 13.5 dpc as detected by X-gal staining and anti-JAM-A staining (Figure 14C and D).

The presence of JAM-A as detected by X-gal staining on the ventral surface of the unturned embryo and in the foregut diverticulum suggests that it is present in the endodermal precursors to the developing mid-gut (Tam et al., 2003). By 9.5 dpc, JAM-A is expressed in the epithelial lining of both the ectoderm component of the oral cavity including Rathke’s pouch (data not shown) as well as the endodermally derived foregut (Kaufman and Bard, 1999) including the nasopharynx, esophagus, and trachea (Figures 11C and 12A) and to a lesser extent the liver (Figure 13C) and pancreas (data not shown). JAM-A gene activity is maintained in the endodermally derived gut epithelium at 12.5 and 13.5 dpc (Figure 14E and F).
Figure 14  Other sites of JAM-A expression at 13.5 dpc are detected by both β-galactosidase activity (A,C,E) and antibody staining (B,D,F). These include the skin and follicles of vibrissae (A,B), the choroid plexus (C,D), and the developing gut tubes (E,F). Abbreviations: cp, choroid plexus; gt, gut tube; pd, periderm; v, follicles of vibrissae. A,C,E: Blue/green indicates JAM-A gene activity; pink is nuclear counterstain. B,D,F: Red indicates JAM-A protein; blue is TO-PRO-3 nuclear counterstain.
Chapter 4
DISCUSSION

An understanding of a protein’s expression during development can provide important insights into its function. As is illustrated in many of the developmental processes described above, JAM-A expression largely correlates to the appearance of intercellular tight junctions. My findings reinforce the importance and prevalence of JAM-A as a tight junction protein. In the pre-implantation embryo, JAM-A expression begins as the morula transitions to the blastocyst. This is coincident with the first sign of tight junctions between cells in the early embryo and the earliest point at which different cell fates begin to develop (Fleming et al., 2000; Fleming et al., 2001). One interesting course of future research would be to explore how JAM-A expression varies as the embryonic stem cells of the blastocyst begin to differentiate. Does the pattern of JAM-A expression at sites of tight junctions continue as the different germ layers begin to form? Are there places in early development where JAM-A is expressed at tissues that do not contain tight junctions? While the role of JAM-A as an adhesion protein is important in many processes, it is also known to be expressed on a number of cell types that do not form tight junctions, such as platelets, neutrophils, monocytes, and lymphocytes (Bazzoni, 2003).

The developing placodal systems in which JAM-A is expressed also seem to show the first signs of gene activity at the time of tight junction formation. The developing inner ear contains tight junctions as early as the otocyst stage (Anniko and Bagger-Sjoback, 1982), around the same point that JAM-A expression begins. The
strong staining pattern of JAM-A in the inner ear has attracted the interest of a group at the National Institutes of Health studying the molecular basis of deafness, and they have been in contact with our laboratory about exploring the role of JAM-A in ear development in greater detail. It has previously been shown that mutations in tight junction proteins can result in deafness in humans (Wilcox et al., 2001), suggesting that this may be a lucrative continuation of my work.

The olfactory system also expresses JAM-A near the time of tight junction formation. Interestingly, the intracellular tight junction associated protein, ZO-1, is expressed in the olfactory placode at 9 dpc (Miragall et al., 1994), roughly two days earlier than JAM-A. ZO-1 is known to associate with JAM-A through its PDZ domain and the extent which JAM-A is responsible for recruiting ZO-1 to the tight junction is unclear. My results support other findings in our laboratory that show ZO-1 is found at tight junctions in the absence of JAM-A (Naik et al., 2003b). It is also notable that ZO-1 expression precedes tight junction formation in the olfactory system (Miragall et al., 1994), again indicating that JAM-A expression correlates to the first sign of tight junctions. JAM-A is known to recruit other proteins to the tight junction, and this function may be necessary for proper tight junction construction and regulation. Tight junctions in the olfactory system are thought to serve as a protective barrier for the olfactory epithelium and olfactory receptor neurons against the harsh environment of the nasal cavity (Hussar et al., 2002), however this potential aspect of JAM-A function has not been explored to date.

Both the lungs and the kidneys also offer a fertile area of future research. The importance of tight junctions in the lungs and their relationship to human airway disease is well established (Montefort et al., 1993; Godfrey et al., 1994). And the
kidneys rely on the interaction between epithelial and endothelial cells—both of which express JAM-A, known to interact in a homophilic manner—in the development of glomeruli (Nikolova and Lammert, 2003). With the JAM-A null mice it would be possible to search for defects in either of these organ systems using a histological approach. The skin and choroid plexuses both provide other examples of tissues where JAM-A expression closely correlates to the initial formation of tight junctions. Tight junctions in the skin arise when the single layer of epithelium forms the periderm and basal layer, which occurs at 9.5 dpc (M’Boneko and Merker, 1988), coincident with JAM-A expression. Similarly, the choroid plexus expresses JAM-A as early as 12.5 dpc, when it is first beginning to form. Tight junctions are present throughout its development (Dziegielew ska et al., 2001).

The expression of JAM-A during vasculogenesis offers valuable insight into its potential role in this process, and in other vascular processes at large. Blood vessel formation involves complicated signaling pathways and several different cell-type transitions. The unique expression pattern of JAM-A surrounding the process of vasculogenesis may provide information about the mechanisms at work. It was observed that JAM-A is broadly expressed early in development, and then becomes primarily localized to the epithelial components of various tissues, including several derived from the mesoderm (such as the kidneys). If this is the case in many tissues, why is JAM-A not expressed during the early stages of vasculogenesis, which involve cells derived from mesoderm epithelia forming into vascular endothelial cells known to express JAM-A once developed?

It is possible that bFGF signaling, which has been shown to cause JAM-A to redistribute to the cell periphery from tight junctions, may be causing a decrease in
JAM-A expression during the epithelial to mesenchymal transition needed for the differentiation of angioblasts from the mesoderm. This transition is associated with a change in cell migration and adhesion, two functions in which the JAM-A-bFGF interaction plays an important role. However, the JAM-A appears to behave differently in epithelial cells compared to endothelial cells. In epithelial-derived breast cancer cells, increasing JAM-A expression leads to the formation of tight junctions and a decrease in cell migration (Naik MU, unpublished data). While in endothelial cells, overexpression of JAM-A causes an increase in cell migration (Naik and Naik, 2006). Based on this, it is tempting to speculate that bFGF secretion from the endoderm signals the epithelial to mesenchymal transition and subsequent migration and differentiation into angioblasts, which occurs partially as a result of decreased JAM-A expression. In the case of early vasculogenesis, a decrease in JAM-A expression mimics the effect of redistributing it from the tight junctions, promoting migration. As the angioblasts gather to form the primitive blood vessels, such as in the allantois at 8.5 dpc, JAM-A expression is still minimal. However, once the blood vessels begin to develop and form definitive endothelial cell-cell contacts, JAM-A expression resumes, as seen in its staining pattern discussed above.

While JAM-A may not be necessary for the “piecing together” of the blood vessel components, it could be vital in subsequent processes, such as angiogenic remodeling. It is known that the intersomitic blood vessels, which correspond to the earliest sign of JAM-A expression in embryonic vasculature, are the first to form in the embryo by angiogenesis (Poole et al., 2001). Thus, the role of JAM-A may be in maintaining endothelial cell adhesion after primitive blood vessel formation as well as promoting subsequent remodeling via angiogenesis. The hypothesis that JAM-A
functions in the later stages of vasculogenesis also correlates well with the observation that antisense inhibition of bFGF or the presence of a dominant-negative form of its receptor (FGFR-1) prevents proper vascular remodeling (Bohsack and Hirschi, 2004). These speculations could be confirmed by a more detailed examination of JAM-A expression during the different stages of vasculogenesis. Co-staining for other proteins known to be found at specific stages of vessel formation could show which processes involve JAM-A. Certainly a greater understanding of how the role of JAM-A varies in different cell types, specifically epithelial and endothelial cells, will shed light on this issue.

In addition to showing that JAM-A is expressed in embryonic blood vessel endothelium, I have shown that JAM-A expression occurs in many sites of epithelial development, including organs derived from all three primary germ layers. Further, my results indicate that JAM-A gene activity often correlates with the first observance of tight junctions in developing tissues, supporting the role of JAM-A as an important tight junction protein and providing novel avenues for future research.
REFERENCES


