CORRELATION OF CHANGES IN MORPHOLOGY AND TGF-β EXPRESSION DURING HUMAN UMBILICAL CORD DEVELOPMENT

by

Ian Copland

A thesis submitted in conformity with the requirements for the Masters of Science Degree, Department of Physiology, University of Toronto

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0-612-50457-3
DEDICATION

This thesis is dedicated:

to my parents, Bill and Susan
for their support and encouragement, and
for helping shape the person I am today.

to my siblings, Heather, Megan and Andrew
for always keeping my ego in check,
and acting as both confidants and advisors.

and, to Jenn
for your love, support, you are my inspiration,
you mean everything to me.
ABSTRACT

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Master’s of Science 2000
Ian Copland
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The umbilical cord is a unique structure that acts as a vascular link between the mother and fetus. The factors regulating its development are poorly understood. In this thesis, the normal morphological umbilical cord development was examined, and related to the expression of the TGF-β isoforms. The expressions of TGF-βs were also examined in umbilical cords from pre-eclamptic pregnancies. The purpose of these studies was to determine what role TGF-β plays in umbilical cord development. Results indicate that TGF-β3 is the main TGF-β isoform expressed throughout gestation, and is significantly decreased in pre-eclamptic umbilical cords. Comparisons with normal umbilical morphological changes suggest TGF-β3 may specifically regulate several events including; vascular smooth muscle differentiation, and Wharton’s jelly collagen deposition, while both TGF-β3 and TGF-β1 appear to influence umbilical endothelial and epithelial morphogenesis. Results suggest that TGF-βs are important regulators of human umbilical cord development.
ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. S.L. Adamson, Dr. B.L. Langille and Dr. S.J. Lye for their supervision and support during my training. Their knowledge and expertise was a tremendous help, I thank them for helping me grow as both a person and scientist.

To Dr. Isabella Caniggia, I thank you for your guidance, support and friendship. To Dr. Kingdom I thank you for your clinical expertise, encouragement and friendship. And to Dr. Martin Post I wish to express my deepest gratitude for your advice, and support, I look forward to working for you in the following years.

To all the members past and present of the Adamson, Lye and Langille labs I wish to extend my sincerest gratitude for their friendship, advice, and teaching during this thesis. I thank each of you individually for contributing to make my Master’s training an enjoyable experience I will never forget.

To Dr. C. Wittnich and the Cardiovascular Collaborative program for broadening my Master’s experience and finally, studentship support from the Medical Research Council of Canada is gratefully acknowledged.
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<tr>
<td>ABC</td>
<td>avidin biotin complex</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyphosphate</td>
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<td>centimeter</td>
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<tr>
<td>DAB</td>
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<td>messanger ribonuclei acid</td>
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<tr>
<td>NBT</td>
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<td>ROD</td>
<td>relative optical density</td>
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<tr>
<td>S.D.</td>
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<td>SDS</td>
<td>sodium disulphide</td>
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<tr>
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<tr>
<td>TBST</td>
<td>tris-buffered saline with tween</td>
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<tr>
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<td>VSMC</td>
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CHAPTER 1: GENERAL REVIEW

The Human Umbilical Cord
1.1 Introduction:

The human umbilical cord is a unique intraamniotic structure that functions as a vascular conduit and represents a lifeline between the mother and fetus. The morphological and structural changes associated with umbilical cord development have been well documented, as are some of the changes in the umbilical environment during gestation. Unfortunately, even with data on the structural changes in several umbilical cord pathologies, very little is known regarding the factors that regulate umbilical cord development. Since the umbilical cord is primarily a vascular structure, I hypothesize that the factors that regulate vascular development will also affect umbilical cord development. Specifically, altered hemodynamics and hormonal levels have the potential to dramatically affect umbilical cord development. The importance of the transforming growth factor betas (TGF-βs) in vascular development and the presence of TGF-β3 in the term umbilical cord also indicates that TGF-β may be important in the regulation of cord development.
1.2 The Human Umbilical Cord at Term:

The umbilical cord has a unique anatomical position, structure and function. Anatomically, the umbilical cord is a free floating intraamniotic structure fixed between two points, the placenta and the fetus. Structurally, it consists of two arteries twisting around a single vein that are surrounded by a matrix-rich structure known as Wharton’s jelly, which is in turn, encased by the epithelium (figure 1.1). The primary function of the umbilical cord is to serve as a vascular conduit through which blood flows to and from the placenta. At the placenta, fetal metabolic by-products are exchanged for a fresh supply of oxygen and nutrients from the maternal circulation. The placenta, specifically the syncytiotrophoblast, is also an important endocrine organ during gestation producing steroid hormones such as estrogen and glucocorticoids (1). The umbilical cord has some unique structural features in comparison to other vascular conduits. Unlike vessels of a similar caliber (e.g. the aorta), the vessels in the umbilical cord contain a relatively large proportion of muscle compared to their collagen content (2), are largely devoid of nerves (3) and are highly reactive to numerous vasoactive stimuli (4). Furthermore, the umbilical vessels must remain widely patent in utero to efficiently conduct huge volumes of blood to and from the placenta, but must also be capable of complete closure immediately after birth (2).
Figure 1.1: The human umbilical cord. Panel a) illustrates the path of the umbilical vessels, where the two umbilical arteries take blood to the placenta and in the cord twist around the single umbilical vein, returning blood to the fetal body. Panel b) illustrates that surrounding the umbilical vessels is a substance called the Wharton’s jelly, which in turn is surrounded by the umbilical epithelium. Modified from Carlson (5).
Since the umbilical vessels are found mostly in the intraamniotic cavity and are often over 60 centimeters in length by term, the cord must protect these vessels from occlusion due to compression, torsion and bending. Ensuring this is the primary function of the Wharton’s jelly, which consists of collagen fibers and an amorphous ground substance composed mainly of hyaluronic acid (6). Together they endow the Wharton’s jelly with a high degree of elasticity and protect the umbilical vessels from compression due to torsion and bending. The main cell type of the Wharton’s jelly is considered a modified fibroblast, because they possess not only the synthetic properties typical of fibroblast, but also contain contractile properties typical of smooth muscle cells. By light microscopy, there is no sharp boundary between the umbilical vessels and the Wharton’s jelly (7;8). Therefore, in addition to protecting the vessels from occlusion, the Wharton’s jelly is believed to serve as a unique common adventitia to the umbilical vessels (9).

The umbilical epithelium resembles the fetal epidermis, but lacks keratinization (10). Functionally, this may allow the umbilical epithelium to be rigid enough to maintain tissue integrity, and to protect the umbilical cord from potentially harmful substances in the amniotic fluid (e.g. infection, toxins). The lack of keratinization suggests the umbilical cord may exchange fluids and nutrients with the surrounding amniotic fluid. This exchange may compensate for another interesting feature of the umbilical cord, the absence of a true vasa vasorum (11).
1.2.1 Umbilical Vessels:

*Endothelium:* Like all other muscular and elastic vessels, the umbilical vessels are lined with a continuous monolayer of flattened, elongated, endothelial cells, that form a barrier between blood constituents and the extravascular space of the vessel wall. Functionally the endothelium is a highly specialized, metabolically-active organ that exerts a multitude of homeostatic effects. Specifically, endothelial cells are involved in the maintenance of vascular integrity, the regulation of the growth and function of surrounding vascular components, and the regulation of vascular tone. The endothelium of the umbilical vessels appears to function similarly to other vessels. Umbilical endothelial cells have the ability to synthesize and secrete extracellular matrix components, such as fibronectin and type IV collagen (12-14), which are important components of the underlying basement membrane. They display cytoplasmic storage bodies called Weibel-Palade bodies, that contain the von Willebrand factor and P-selectin proteins (15;16) required for the activation of endothelial cells. As well, they display the usual system of interendothelial cell junctions, including gap junctions (17;18), and have the ability to regulate vascular tone and contraction (19) through their influences on vasoreactivity (20;21).

*Media:* The media of the umbilical vessels is composed primarily of vascular smooth muscle cells, elastin, collagens, and proteoglycans. In the umbilical arteries, the smooth muscle cells can be divided into two distinct regions, an outer region and an inner region. The outer region consists of tightly packed, well-defined, circularly arranged smooth muscle cells, mixed with rows of longitudinal smooth muscle cells. The inner region consists mainly of smooth muscle cells that are loosely arranged, have an obscure
orientation and poorly defined cellular outlines. Within the umbilical vein, smooth muscle cells display features similar to those in the outer region of the umbilical arteries (22).

At term ultrastructurally, the smooth muscle cells of the outer region appear identical to the mature contractile smooth muscle cells found in other muscular arteries (22;23). They display an abundance of thin filament bundles, numerous pinocytotic vesicles, and continuous basement membranes. Cavaille et al. (24) also showed that term umbilical vessels display numerous contractile protein isoforms typical of mature vascular smooth muscle cells. Thus, vascular smooth muscle cells of the outer region in the umbilical vessels appear phenotypically mature and mechanistically capable of generating contractions. In contrast, smooth muscle cells in the inner region, ultrastructurally, contain few thin filament bundles, have few pinocytotic vesicles and display gaps in their basement membranes (8). The cytoplasms of the inner smooth muscle cells are occupied by numerous synthetic organelles such as a well developed, dilated, rough endoplasmic reticulum and a prominent Golgi apparatus. Thus, the smooth muscle cells of the inner layer in umbilical arteries likely have a biosynthetic (25) rather than contractile role. These cells may also serve as a plastic tissue component to fill the narrowing arterial lumen at birth and thereby, contribute to the later stages of umbilical artery closure (22).

Elastic tissues are present in all vessels, with the exception of capillaries. Functionally, elastin is the main passive component that gives blood vessels their elasticity. In small vessels, elastin is usually exclusively located in a specialized layer called the internal elastic lamina, which lies adjacent to the endothelium and separates the
smooth muscle cells from the endothelium. In larger vessels, in addition to an internal elastic lamina, secondary elastin bands are often found circularly orientated throughout the vessel wall. For these larger vessels, elastin may account for a significant proportion of the total wall weight, and in the aorta, elastin accounts for as much as 40% of the total vascular wall weight (26). In the umbilical arteries, elastin accounts for 26% of the total vessel wall weight (27). However, unlike aortic elastin, umbilical vascular elastin does not form complete elastic laminas, resulting in a fragmented appearance (22);(2). The precise function of umbilical elastin is currently unknown, although a similar pattern of elastin fragmentation in the ductus arteriosus has been associated with cellular migration (28).

Collagen is a group of at least 14 distinct proteins (29). Typically, collagens are composed of three polypeptides chains (called α chains) wound around each other to confer the characteristic stiff, triple helical structure (30). Vessel walls themselves consist mainly of type I, III, IV and V collagens (31);(32). Collagen types I and III function as structural (fibrillar) collagen which, when exposed to increasing stretch, straighten to become stiff and prevent over-distension of the vessel (33). Type IV collagen functions as a scaffold for the assembly of other basement membrane components and endows the basement membrane with a size-selective filtration property (34). Type V collagen forms heterotypic fibrils with types I and III collagens in blood vessels, which helps organize collagen fibrils. Studies in the human aorta, carotid arteries and pulmonary arteries consistently indicated type I collagen as the major type present (35-37). Type I collagen is also the most abundant collagen in the umbilical arteries (63.5% of the total collagen) (38). However, unlike elastin, total arterial collagen content is
only 13% of total wall weight which is dramatically lower than that of the aorta at 24% collagen (26). Therefore, although the umbilical arteries contain both type I and III collagen, their low total collagen content suggests that these vessels likely depend more heavily on their smooth muscle content than their collagen content to prevent over-distension.

Proteoglycans (PG) are a diverse family of molecules characterized by a core protein attached to one or more glycosaminoglycan side chains (39). In the extracellular milieu, glycosaminoglycans, because of their large physical size, concentration and ionic properties, influence processes like tissue permeability, filtration, and ion exchange (40). Glycosaminoglycans in the umbilical vascular wall include chondroitin sulphate, heparan sulphate, dermatan sulphate and keratan sulphate (39), typical of most vessels. However, unlike most vessels, hyaluronic acid is the most abundant glycosaminoglycan in the umbilical arteries, comprising 40% of the total glycosaminoglycan content (41) compared to 4-5% in the aorta (42).

1.2.2 The Wharton’s jelly.

The Wharton’s jelly of the human umbilical cord is a mucous connective tissue that binds and encases the umbilical vessels (43). It is composed primarily of collagen fibers and modified fibroblasts embedded in an abundant amorphous ground substance rich in hyaluronic acid and proteoglycans (44). Generally, these fibroblastic cells are more densely packed in the vicinity of the vessels than near the epithelium. These cells are mainly stellate-shaped with long cytoplasmic processes and are described as modified fibroblasts (6;9). They contain numerous synthetic organelles, typical of active
fibroblasts, yet also contain numerous gap junctions and a basement membrane-like substance on their cell surface typical of smooth muscle cells (6). Consequently, based on their fibroblastic nature, these cells possess the ability to synthesize collagen and other extracellular matrix proteins, whereas their smooth muscle characteristics may allow these cells to produce active tension.

The collagen within the Wharton's jelly acts as a complex interlacing network, that provides a scaffold that influences the spatial organization of cells and the distribution and storage of ground substance and water. It also controls the movement and growth of the umbilical vessels. Collagen in the Wharton's jelly is made up primarily of type I (47%) and type III (45%) collagens, which comprise more than 50% of the dry weight (41). Collagen fibers are organized into a continuous spongy network, characterized by non-random alternating wavy bundles anchored by reticular fibrils (43). The network around the vessels is compact, but under the umbilical epithelium it is characterized by numerous empty spaces that create a wide system of interconnected cavities (43).

1.2.3 Epithelium:

Surrounding the Wharton's jelly, and encasing the entire umbilical cord, is an epithelial lining derived from the amniotic epithelium. In the rat, this epithelial lining is morphologically similar to the amniotic epithelium (45) however, the umbilical epithelium of humans more closely resembles the fetal epidermis (46). At term, the umbilical epithelium is composed of between one and five layers of flattened cells separated from the Wharton's jelly by a thick, irregular basement membrane. Studies by Plentl (47) and Hutchinson (48) suggested that umbilical epithelium is involved in the
direct exchange of fluid between the cord blood and the amniotic fluid. Numerous villous folds, in the intercellular spaces, between superficial cells of the epithelium indicate the possibility of an uninterrupted diffusion pathway across the umbilical epithelium (10).

1.2.4 Distended and Nondistended Human Umbilical Cords:

The conventional picture of the cross-section of the umbilical cord indicates that the area occupied by Wharton’s jelly is much greater than the area occupied by the blood vessels. By simultaneously clamping two points of the cord, while blood flows freely, observations of the umbilical cord in a relatively distended state can be obtained (figure 1.2). The principle finding is that in the distended cord, the amount of Wharton’s jelly is greatly reduced, appearing as a thin membrane surrounding and separating the three enormously distended blood vessels (49). Furthermore, the thickness of the distended artery wall is about one-seventh of that of the constricted artery, while in a distended vein, the wall is about one tenth the thickness of the constricted vein wall (50). Thus, in histological studies, researchers must be aware that, morphologically, umbilical cords are very different ex-utero versus in-utero.
Figure 1.2: Cross-section of the human umbilical cords at term. The upper cord (a) shows the appearance of the collapsed umbilical cord, while the lower cord (b) is from a cord that has been clamped off to maintain its blood pressure. Comparison between the two shows that the collapsed cord has a reduced intravascular volume, and a greatly increased Wharton’s jelly area. Modified from Reynolds (49).
1.3 Development of the Umbilical Cord:

The previous section demonstrates that a considerable amount of information is available on the structure of the term umbilical cord. In this section I will review what is known about the embryogenesis of this complex structure and outline some of the developmental changes associated with normal umbilical cord development.

1.3.1 Embryonic Period

The primitive umbilical ring first forms as the embryonic disc flexes and folds. Through this ring extends the connecting stalk (a mesenchymal structure that bridges the exocoelom and lies basal to the amniotic vesicle) followed by the allantois, yolk-sac stalk, vitelline vessels and the extraembryonic coelomic cavity (51) (figure 1.3). Around the fourth week, as the dorsal aortas undergo fusion, the definitive umbilical arteries appear as two lateral branches originating from the caudal end of the dorsal aorta. These arteries extend into the primitive umbilical cord, and unite with the allantoic artery, forming an interarterial anastomosis near the umbilical cord placental insertion (52). The umbilical vein first appears as two separate vessels around five weeks, with the right umbilical vein usually regressing by eight weeks (53). By ten weeks, the obliteration of extraembryonic coelom and regression of the allantois and vitelline vessels (54) and signals the end of the umbilical cord’s embryonic period.
Figure 1.3: The formation of the human umbilical cord. Between 3.5 and 4.5 weeks gestation, the allantosis, yolk-sac stalk, and portions of the extraembryonic coelomic cavity invade or are incorporated into the connecting stalk. Shortly afterwards this structure becomes vascularized. The definitive umbilical cord is formed when the expanding amnion compresses all these components into a slender cord-like structure. Modified from Carlson (5).
1.3.2 Fetal Period:
Once the definitive umbilical cord has formed, each component must grow, and differentiate in order to assume their physiologic functions. In the umbilical vessels, internal luminal diameter increases 3-4 fold from 15 weeks to 40 weeks for both the arteries (1.2- 3.9 mm) and vein (2.0- 7.8 mm) (55). Each vessel also develops a thicker media with concurrent increases in smooth muscle cell myofilament content and ratio of contractile to synthetic smooth muscle cell phenotype (7). The total area of Wharton’s jelly increases approximately 12-15 fold, between 10 weeks and term (55), and occurs simultaneously with the maturation of the fibroblast cells (56), deposition of hyaluronic acid (57) and collagen fiber formation (58). During gestation, the umbilical epithelium progresses from a single layer of cuboidal undifferentiated cells to a multi-layered structure at term. Ultrastructurally, the umbilical epithelium appears to be impermeable to water until the 6th month of gestation when the disappearance of the zonulae occludentes (10) suggests an uninterrupted pathway for fluid diffusion across the epithelium.

Aside from morphological changes, numerous extrinsic changes also occur within and around the umbilical cord in the fetal period. For instance, prior to 10 weeks placental pO₂ levels are lower than endometrial levels (59;60). Yet with the establishment of a continuous maternal blood flow in the intervillous space the placenta changes from a relatively hypoxic to a normoxic tissue (61) thereby facilitating the increase in fetal blood pO₂. The placenta is also responsible for producing large amounts of sex steroids including estrogens, as well as numerous polypeptide hormones (e.g. cortisol), which together play a major role in maintaining pregnancy and promoting fetal growth and well-being (1). The concentrations of estrogen and cortisol in both the fetal circulation and amniotic fluid increase during gestation (62-67). Therefore, since the
umbilical cord is in direct contact with the fetal circulation and amniotic fluid, alterations in PO₂ and hormone levels may influence cord development.

As pregnancy progresses, fetal intravascular hemodynamics change. In humans, umbilical blood flow gradually increases as the fetus grows (68). During gestation umbilical blood flow increases tenfold from the 12th to the 28th week of gestation (69), and at term, comprises approximately 30% of the fetal biventricular cardiac output (70). Increasing blood flow is also accompanied by increases in umbilical arterial and venous blood pressure (71;72), and by term are approximately 50 and 25 mmHg respectively (73). Consequently, mechanical stresses associated with vascular hemodynamics (shear stress, tangential wall tension) changes during fetal development may result in load specific tissue remodeling (74) in the umbilical cord.
1.3.3 Pathologies of the Human Umbilical Cord:

At term and during development several umbilical cord abnormalities can have a bearing on fetal survival and prenatal oxygenation. Several of these abnormalities will be the topic of this section.

**Length:** Numerous studies have reported that normally the umbilical cord grows in a linear fashion during gestation. Abnormally long cords are associated with cord entanglement, prolapse and hematomas (75), while abnormally short cords predispose the fetus to rupture of the cord, failure to descend in labor, abruption of the placenta (76). Consequently, abnormal umbilical cord length is a risk factor for adverse perinatal outcome, although currently the measurement of umbilical cord length is not widely applied in clinical obstetrics.

**Single Umbilical Artery:** A single umbilical artery is associated with fetal anomalies (77,78) and is linked to an increase in perinatal morbidity and mortality. Umbilical cords with a single umbilical artery show increases in arterial luminal diameter and total vascular area (79). These changes themselves do not result in increased fetal morbidity and mortality but rather act as sonographic diagnostic markers for perinatal structural, and chromosomal abnormalities.

**Pre-eclampsia:** Pre-eclampsia is a disorder specific to pregnancy, recognized by maternal hypertension with proteinuria or edema, or both (80). Pre-eclampsia is the major cause of maternal and fetal mortality and morbidity in developed countries (81)
and is associated with abnormal placental development due to shallow invasion of the maternal decidua by trophoblast (82). The incidence of pre-eclampsia ranges between 7-10% of all pregnancies (83) and when severe is associated with coexistent fetal growth restriction (82;82). Pre-eclampsia is also associated with abnormalities in umbilical hemodynamics (84), and hormone levels (85-88).

Pre-eclampsia is associated with a doubling of collagen content in umbilical arteries, an increase in the proportion of type III collagen and a significant decrease in elastin content (27;38). In both the arteries and the Wharton’s jelly, a premature reduction in hyaluronic acid is also associated with pre-eclamptic umbilical cords (89;90). Consistent with these measurements, pre-eclamptic umbilical arteries also show changes in their passive mechanical properties (91), in which collagen and elastin are the main components (92).

Intrauterine growth restriction (IUGR): The risk of perinatal morbidity and mortality is increased in growth restricted fetuses, in proportion to the severity of the growth restriction. A fetus is categorized as growth restricted when its birth weight is less than the 10th percentile for its gestation (93). Presently, research suggests that genetic factors, infection/toxin, multiple gestation, maternal nutrition, placental and hormonal factors, as well as fetal cardiovascular anomalies and uterine placental perfusion all contribute to growth restriction (93). The general finding in umbilical cords from growth restricted pregnancies is a reduction in total umbilical cord area due to decreases in total vessel and Wharton’s jelly area (94).
Gestational Diabetes: The incidence of gestational diabetes is between 3.2-5% (95) of all pregnancies, and is characterized as a carbohydrate intolerance during pregnancy, that resolves itself after delivery (93). Sonographically, umbilical cords from mothers with gestational diabetes are significantly enlarged due to an increase in Wharton’s jelly content (96). Diabetic umbilical cords also show alterations in endothelial integrity, smooth muscle organization, and collagen (97).

1.3.4 Regulation of Human Umbilical Cord Development:

In the previous two sections, I outlined changes that occur in the human umbilical cord during normal and abnormal development. At present, the factors that regulate umbilical cord development are unknown. However, since the umbilical cord is composed of three large vessels, the factors that regulate vascular development likely play important roles in umbilical cord development.

1.4 Regulators of Vascular Development:

There are two separate processes involved in vascular development; 1) the establishment of a primary vascular network comprised of a nascent endothelial cell tubes and 2) the morphogenesis of the vascular wall. This review will only consider the regulation of vascular wall morphogenesis, since my studies are concerned with maturation rather than the establishment of the umbilical cord.

Despite considerable amounts of literature describing neovascular processes in wounds, tumors and pathologies, the mechanisms that regulate vessel wall morphogenesis, in vivo, are poorly understood. Major deficiencies still exist in our
understanding of vascular smooth muscle cell maturation in vivo due to a lack of specific cellular and molecular markers, and uncertainty with regards to relevant regulatory molecules (98). Consequently, the search for regulators of vascular smooth muscle cell maturation has been largely accomplished using an in vitro approach. Cell culture studies have helped define a number of extrinsic factors and local environmental cues believed to be important in controlling the differentiation/maturation of the vascular smooth muscle cell (99-102), and are summarized in figure 1.4. This figure illustrates that differentiation/maturation of smooth muscle cells is dependent on the complex interaction of multiple local environmental signals, and that modification of any one of these factors may contribute to an alteration in the differentiated state of the smooth muscle cell (i.e., phenotypic modulation). In the following subsections I will briefly review how individually mechanical forces, hormones and local diffusible factors can influence vascular wall morphogenesis.
Figure 1.4: Summary of extrinsic factors and local environmental cues believed to be important in controlling the differentiation/maturation of the vascular smooth muscle cell (SMC). The smooth muscle cell can exhibit a wide range of different phenotypes ranging from the highly synthetic proliferative cell on the left to the mature, fully contractile cell on the right. The multiple arrows indicate that a range of phenotypes are possible between the two extremes and that change appears to be reversible. Modified from Owens (98)
1.4.1 Mechanical Forces:

In vitro mechanical stretching simulates the effects of increased blood pressure and, when imposed on vascular smooth muscle cells, causes reorientation and differentiation of these cells (103-105), increases protein and DNA synthesis (106;107), and stimulates the production of extracellular matrix proteins (108-110). In vivo, the establishment of blood flow coincides with the incorporation of smooth muscle cells into developing arteries, whereas increased blood pressure and pulse pressure correlate with the expression of smooth muscle cells differentiation markers (111;112) and matrix development. In vascular tissues exposed to long-term changes in hemodynamics, structural remodeling is specific to the type of hemodynamic load altered. Increased flow increases vessel lumen diameter, while increased pressure elicits a thickening of the vascular wall (74). Based on these data, I hypothesize that the increases in luminal diameter and vascular wall thickness seen during umbilical cord vascular development are influenced by altered umbilical mechanical forces secondary to gestational increases in blood flow and pressure.
Hormones, in particular the sex steroid hormones, can affect multiple aspects of vascular wall morphogenesis. In the endothelium, estrogen increases the availability of vasodilatory factors like endothelium-derived nitric oxide (113) and prostacyclin (114), while also decreasing vascular responsiveness to vasoconstrictive agonists (115). In cultured vascular smooth muscle cells, estrogen reduces proliferation (116;117) and collagen production (118;119). Glucocorticoids also have the ability to affect vascular structure and function (120;121). Like estrogen, glucocorticoids can inhibit cell growth of cultured vascular smooth muscle cells (122); however, in contrast to estrogen, glucocorticoids enhance the contractile response of known vasoconstrictors (123) and can be directly synthesized by vascular tissue (124). Consequently, the increase in both estrogen and glucocorticoid levels in the fetal blood and amniotic fluid during gestation may have a dramatic influence on umbilical cord development.
1.4.3 Influence of Local Factors in Vascular Morphogenesis:

The milieu of the developing vasculature contains a rich assortment of local factors that can influence vascular morphogenesis. In vitro studies have identified roles for several growth factors in endothelial and smooth muscle cells, however these roles have not been firmly established in vivo. In the following section, I will outline the influences of several growth factors on vascular wall morphogenesis and outline a model for vascular wall assembly that incorporates the functions of several of these growth factors.

**Insulin-like Growth Factors:** The insulin-like growth factors (IGF-I and IGF-II) are about 60% homologous with insulin (125) and are general growth simulators. IGF-I and IGF-II both have mitogenic effects on aortic smooth muscle cells (126) and stimulate directed migration of smooth muscle cells in a gradient of IGF (127).

**Platelet Derived Growth Factor:** Platelet derived growth factor (PDGF) is a potent mitogen for vascular smooth muscle cells. PDGF-like molecules are known to be synthesized by endothelial cells, smooth muscle cells and activated macrophages (126). In addition to its mitogenic effects, PDGF is chemotactic for a variety of cells, including vascular smooth muscle cells (128). In situ hybridization shows that PDGF receptors are present in the 10.5 day embryonic mouse vessels (129) and when inactivated, by targeted gene mutation, vessels become abnormally dilated apparently due to defective cellular migration and vascular smooth muscle cell contractility (130).
Angiopoitein: Angiopoitein is a 70 kD glycoprotein, that is the first known ligand of the TIE2 receptor (131). Unlike IGF and PDGF, angiopoitein is not a mitogen, but rather appears to play a role in regulating the assembly of non-endothelial vessel wall components (132).

Transforming Growth Factor Betas (TGF-βs): The term “TGF-β” is used to denote a family of multifunctional growth factors which regulate cell proliferation, differentiation, and the expression of extracellular matrix (ECM) proteins (133). Detailed analyses of TGF-β isoform mRNAs and protein localization’s have revealed distinct patterns of expression during murine vascular development. With respect to the developing vasculature, the principal isoform expressed is TGF-β1. TGF-β1 mRNA first appears at day 7 post coitum (p.c) in the blood islands of the yolk sac. Within the embryo proper, TGF-β1 mRNA expression is seen in the endothelium of all major blood vessels by day 9.5 p.c. (134). Neither TGF-β2 or β3 are expressed in the yolk sac, however, TGF-β3 is often expressed in the both tunica intima and tunica media of major blood vessels, while TGF-β2 is usually expressed in the tunica media (135). Analyses of TGF-β knockout mice indicate that TGF-β1 is critical in maintaining vascular endothelial integrity, since in the knockout animals defective endothelial differentiation results in inadequate capillary tube formation and weak vessels (136). The phenotypes of TGF-β2 and TGF-β3 null mutant mice, on the other hand, imply significant roles in mesenchymal- epithelial interactions, cell growth and migration and matrix formation, (137-139).
Model for Vascular Wall Morphogenesis: A proposed model by which TGF-β influences vascular assembly and wall morphogenesis is seen in figure 1.5. In this model, angiopoietin-1 produced by mesenchymal cells activates the TIE2 receptor on endothelial cells. This leads to the production and or release of a recruiting signal for mesenchymal cells. Most data indicates that for smooth muscle cells this signal is PDGF. Once mesenchymal cells arrive in contact with the endothelium, TGF-β may be activated. The TGF-β may then induce differentiation of the mesenchymal cells into smooth muscle cells.
**Figure 1.5:** Proposed Model for Growth Factors in Vascular Wall Morphogenesis. Angiopoietin released by mesenchymal cells binds to the TIE-2 receptor and activates a signal from the endothelial cell that recruits local mesenchymal cells to the developing vessel. Upon contact with the endothelium, TGF-β is activated (released) causing mesenchymal cells to differentiate into smooth muscles cells, inhibiting endothelial proliferation, and promoting the accumulation of extracellular matrix. Modified from Folkman et al., (140).
1.5 Growth Factors in the Umbilical Cord.

In the term human umbilical cord, several growth factors have been identified including; IGF (141), EGF (142) and TGF-β (143; 144). In particular, the level of TGF-β3 mRNA in term umbilical cord extracts shows one of the highest levels of expression of any tissue studied (145). TGF-β3 protein is localized to the vascular smooth muscle, endothelial, epithelial and fibroblastic cells, and is identical to recombinant TGF-β3 (143). TGF-β3 appears to be the predominant TGF-β isoform at term, however whether it predominates during early and mid-gestation is unknown. In the following sub-sections I will highlight the properties of TGF-β3 in relation to the other mammalian isoforms of TGF-β and discuss the effects that gestational changes in umbilical cord hemodynamics and hormonal balance may have on umbilical TGF-β expression.

1.5.1 TGF-β Isolation, Structure, Receptors and Signal Transduction:

Transforming growth factors were first detected in cells transformed by murine and feline sarcoma viruses (146;147). TGF-β was identified by its ability to elicit anchorage-independent growth and transform the phenotype of mesenchymal cells. TGF-β has since been included in a superfamily of growth differentiation and morphogenesis factors (148). To date five isoforms have been identified for the TGF-beta family in vertebrates of which type 1, 2, and 3 are in present in mammalian species.

The mammalian isoforms of TGF-B 1, 2, and 3, are localized to three different chromosomes (human chromosomes 19q13, 1q41, and 14q21,respectively). The genes encoding each TGF-β isoforms have seven exons and intron-exon splice junctions (133),
but individually each isoforms shares very little homology in their promoter regions (149).

When translated, a TGF-β3 protein shares approximately 80% and 83% sequence identity with TGF-β1 and TGF-β2 respectively (150) and like other TGF-βs, the bioavailability of TGF-β3 can be regulated by accessory proteins. Specifically, TGF-β isoforms are synthesized as part of a larger precursor molecule, in which the carboxy terminal is proteolytic cleavage of the amino-terminal pro-domain. Once cleaved two carboxy terminal TGF-βs associate and fold into the mature TGF-β protein. The cleaved amino-terminal pro-domain helps with this carboxy protein folding and confers a degree of latency on mature TGF-βs through noncovalent interactions. The amnion pro-domain is thus referred to as the latency associated protein. Newly synthesized TGF-βs are primarily secreted this latent form and must be activated before they can interact with TGF-β membrane receptors. In addition latent TGF-βs themselves can also associate with, latent TGF-β binding proteins (151) which can target latent TGF-βs to the extracellular matrix allowing the formation of TGF-β reservoirs (see figure 1.6). Thus, aside from transcriptional TGF-βs can also experience two separate levels of post-translational control.
Figure 1.6: TGF-β protein structure. Panel a) illustrates the TGF-β protein structure prior to post-translational modification secretion. Each peptide contains a mature C-terminal portion and a latent N-terminal portion. The two portions are cleaved from one another (arrow) intracellularly. This allows two C-terminal portions to join prior to secretion, while the latent portion folds around C-terminal peptide masking their biological activity and allowing the complex to specific extracellular matrix binding proteins (b).
Active TGF-βs exert their functions by binding to specific receptors including receptors type I, type II, betaglycan and endoglin (152-155). Among these the serine-threonine kinase receptors types I and II are necessary for all biological responses and are ubiquitously expressed in most tissues (156). Initially, the TGF-β ligand binds to the type II receptor, which is an invariably active serine/threonine kinase. Bound TGF-β is then recognized by the type I receptor, which is recruited into the complex and becomes phosphorylated by the type II receptor (157). This phosphorylation activates the type I receptor enabling it to signal to downstream targets (Smads), which in turn translocate to the nucleus and influence cellular gene expression (158). Betaglycan and endoglin both form heteromeric complexes with the signaling receptors type I and II and modulate the cellular responses of TGF-β ligands (159). Following binding, the resulting ligand-receptor complex is internalized and degraded by lysosomal enzymes. Currently no study has identified the TGF-β receptors or signal transducing molecules in the umbilical cord of any species.

1.5.2 Mechanical and Hormonal Regulation of TGF-βs:

In vitro, TGF-β expression can be altered by changes in shear stress (107;160;161) and stretch (162;163) in endothelial, vascular smooth muscle and mesenchymal cells. In vivo, altered TGF-β expression is associated with pathologies like hypertension (164) where hemodynamic forces can be altered. Thus, the gestational increases in umbilical blood flow and blood pressure may influence the gestational expression pattern of TGF-βs in the cord. Gestational changes in hormone levels, within and around the umbilical cord, may also have an effect on TGF-β expression.
Specifically, TGF-β expression is influenced by estrogens (165) and glucocorticoids (166). Therefore, since both estrogen and glucocorticoids (62-67) increase in the fetal blood and amniotic fluid during pregnancy, TGF-β expression may also be hormonally regulated in the developing umbilical cord.
1.6 Rationale:

Presently very little is known regarding the regulation of umbilical cord development. Alterations in hemodynamics and hormonal levels are both potentially important regulators of cord development. However directly measuring fetal hemodynamics or hormones and correlating their gestational changes with morphological changes in the human umbilical cord would be technically very difficult. Thus, a good first step in understanding regulation of umbilical cord development is to look for gestational changes in a local regulator and relate that to morphological changes. Previous research has demonstrated TGF-βs are found throughout vascular development and in the term umbilical cord, the TGF-β3 isoform is highly expressed on both a protein and mRNA level. Presently the gestational expression patterns of the mammalian TGF-β isoforms have not been characterized. It is, therefore, the purpose of this thesis to characterize the expression pattern of the TGF-βs throughout umbilical cord development and correlate their expression with specific morphological changes during normal development. This thesis also examines whether TGF-β expression is altered in pre-eclamptic umbilical cords, in order to provide additional clues as to specific TGF-β isoform functions in vivo.
1.7 Objectives:

The specific objectives of this thesis were to:

1. Examine the time course of morphological changes during normal human umbilical cord development (Chapter 2).

2. Determine the gestational expression profiles for the mammalian TGF-β isoforms during normal umbilical cord development (Chapter 3).

3. Compare total TGF-β protein expression between normal and pre-eclamptic umbilical cords (Chapter 3).

4. Correlate changes in TGF-β expression with normal developmental changes in human umbilical cord development (Chapter 4).

5. Correlate changes in pre-eclamptic TGF-β expression with alterations in structure, hemodynamics and hormonal balance (Chapter 4).
CHAPTER 2:

Morphological Changes in Normal Umbilical Cord Development
2.1 Introduction

The structural development of individual umbilical cord components has been the subject of considerable research (7; 10; 55-57; 167-169). However, apart from Milani, there has been little attempt to follow the development of the umbilical cord as a whole (see Leeson ref. # (168)). The present study is concerned with the changes in gross histological structures in the human umbilical cord, from 6 weeks until term (~40 weeks).
2.2 Material and Methods

2.2.1 Sample Collection and Fixation:

Human umbilical cord samples were obtained following elective termination during the first (6wk-12wk, N = 7) and second trimester (13wk-16wk N = 7; 17-20wk, N=8), and following vaginal or cesarean section delivery in the third trimester (32wk-40wk, N = 10). Within 30 minutes of delivery umbilical cord samples were washed in phosphate buffered saline (PBS pH 7.4) to remove adherent blood. Normally, three 1-cm cross-sectional segments were obtained for histology, one from the middle portion of the cord, and one from either end. Light microscopy was performed primarily on the middle portions to reduce inconsistencies since most cords were not attached to the fetus or placenta. Once cut, attempts were made to remove blood from within the lumen of vessels by pulling out coagulated blood from the cut ends of the vessels with fine forceps. Segments were then fixed by immersion in 10% neutral buffered formalin for 48 hours at 4°C.
2.2.2 Embedding, Sectioning and Staining:

Fixed cord segments were taken to the Pathology Laboratory at the Hospital for Sick Children (Toronto, Ont.) for embedding, sectioning and staining. Cord segments were dehydrated in an ascending ethanol series, cleared in xylene, then embedded in paraffin. Five-micron transverse sections of the wax embedded specimens were cut and placed on SuperFrost Plus slides (Fisher brand), dried overnight at 37 °C, and stored at room temperature until stained.

Prior to Movat’s pentachrome staining, all sections were dewaxed in xylene and rehydrated in a descending ethanol series. Sections were then placed in Bouin’s solution for one hour at 60°C, washed in running water, rinsed in 1% acetic acid, and stained in alcian blue for five minutes. Sections were again rinsed in 1% acetic acid, washed in water and for 9 minutes sections were immersed in an elastin stain. Excess stain was removed by washes in running water, before staining the sections in Biebrich scarlet-acid fuchsin for 1 _ minutes. Sections were then washed in water, rinsed in 0.5% acetic acid and allowed to differentiate in phosphotungstic acid for five minutes. Finally sections were rinsed in 0.5% acetic acid and three changes of absolute alcohol, before being stained in saffron for 5 minutes, dehydrated, cleared and mounted (170). After staining, nuclei appeared purple, cytoplasm red, collagens yellow, elastin black and proteoglycans blue/green.
2.3 Results

2.3.1 Morphological changes:

*Endothelium*:

Throughout gestation, the endothelium of both the umbilical vein and arteries remained as a single layer lining the lumen of the vessels. From mid to late gestations the nuclei (purple) of endothelial cells protruded into the lumen of the vessel, particularly in regions of contraction (figure 2.1).

*Vessel Wall*:

During gestation, there was a progressive increase in thickness of the both the umbilical arterial and venous walls (figure 2.2) that occurred concomitantly with increased contraction of the vessels (figure 2.3). The umbilical arterial walls were thicker than the wall of the vein throughout gestation (figure 2.2). Between 6 and 15 weeks, the general arrangement of the smooth muscle layers (red) were similar in the arteries and vein (figure 2.2 a-d); however, the appearance and growth of a distinct inner region of the umbilical arterial wall, after 16 weeks, distinguished the umbilical arteries from the vein. This difference was first demarcated by differences in elastin deposition (black staining) between the arteries and vein. Between 14-16 weeks, elastin first appeared as a single thin discontinuous band between the endothelium and vascular smooth muscle in both the arteries and vein (figure 2.4 c,d). Later in gestation, additional bands of elastin spread out from the lumen of the umbilical arteries (figure 2.4 e,g) and, by term, elastin was no
longer seen between the endothelium and innermost layers of smooth muscle. Instead an abundance of proteoglycan staining (blue/green), was seen at this location. In contrast to arterial elastin deposition, venous elastin remained a single band separating the endothelium from the vascular smooth muscle (figure 2.3 g, 2.4 g). This band increased only in thickness between 20 weeks and term (figure 2.4 f, g).

*Wharton’s Jelly:*

Collagen staining (yellow) increased throughout gestation in the Wharton’s jelly (figure 2.5). Collagen was identified at 8 weeks, but primarily consisted of scattered, unorganized, individual collagen fibers. From 10 weeks onwards, collagen gradually increased in content and reorganized, so that thick bundles could be identified throughout the Wharton’s jelly by term (figure 2.5 a-d). Collagen fibers were generally more dense around the vessels than under the umbilical epithelium (figure 2.5 f). The fibroblasts of the Wharton’s jelly were generally stellate shaped (figure 2.5 a-d) and, like collagen, were found at a higher density around the vessels than under the umbilical epithelium (figure 2.5 e).

*Umbilical epithelium:*

During gestation, the umbilical epithelium became more complex. It increased from a single layer of cells (red) before 12 weeks (figure 2.6 a) to a multilayered structure between 15 weeks and 20 weeks (figure 2.6 b). By term, the epithelium consisted of as many as 5 layers (figure 2.6 c), but could range from 2 to 5 layers (figure 2.6 d) within individual sections and between samples.
**Figure 2.1:** The umbilical endothelium in the artery and vein during gestation. In the artery shown on the left (a,c,e) and the vein shown on the right (b,d,f), the endothelium is a single layer lining the lumen (L) throughout gestation. Individual cells (red) were generally flattened and elongated early in gestation, but individual endothelial nuclei (purple) frequently protruded into the lumen of both the artery (e) and vein (f) later in gestation. Magnification 1000X.

SM = smooth muscle, WJ = Wharton’s jelly.
Figure 2.2: Vascular wall development of the umbilical artery and vein during gestation. During gestation there was a progressive increase in wall thickness of both the artery (a, c, e, g) and vein (b, d, f, h). Between 6 weeks and 15 weeks, the changes in the vascular structure, of the artery (a, c) and vein (b, d) were similar. An inner region (marked by double headed arrows) within the umbilical arterial wall became well-defined between 20 weeks (e) and term (g). This region was characterized by increased proteoglycan staining (blue/green) and was bounded by numerous bands of elastin (black) (g).

Magnification, 6 wk (a,b) X1000, 15 wk (c,d) X400, 20 wk (e,f) X 200, term (g,h) X 100.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s jelly.
**Figure 2.3**: Contraction of the umbilical artery during gestation. Throughout gestation, the umbilical vessels became increasingly contracted (a-d). At 12 weeks the arterial vascular lumen was fully patent and displayed a circular lumen (a). Between 15 and 20 weeks, the arterial vascular lumen was still largely unobstructed, however local protrusions of the endothelial and vascular smooth muscle cells in the lumen changes it shape from circular to bulbous (b,c). By term, the lumen of the artery was almost completely obstructed and star-shaped (d).

Magnification a 400 X, b 200 X, c 100 X, d 100 X.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s jelly.
**Figure 2.4:** Elastin deposition in the umbilical cord during gestation. Prior to 15 weeks, elastin could not be identified in either the umbilical artery (a) or vein (b). Between 14-16 weeks, elastin (black) was seen in both the umbilical artery (c), and vein (d), as a single band (indicated by arrows) discontinuously separating the endothelium from the vascular smooth muscle. From 15 weeks on towards term (e, g), additional fragmented bands of elastin appeared further from the arterial lumen between the smooth muscle layers. By term, elastin no longer separated the endothelium from the vascular smooth muscle (g) in the artery. In contrast, venous elastin appeared unchanged between 15 (d) and 20 (f) week, and only increased in thickness between 20 (f) weeks and term (h).

Magnification a-f X 1000, g-h X 400.

L = Lumen, SM = Smooth Muscle.
Figure 2.5: Collagen deposition in the Wharton's jelly, during umbilical cord development. At 8 weeks (a) collagen was difficult to distinguish, but by 15 weeks (b) collagen (yellow) was seen as scattered fibers and small bundles. Between 15 weeks and term (b-d) collagen increased in both content and organization and was generally denser around the vessels than under the umbilical epithelium (f). Throughout gestation, the fibroblasts (red) in the Wharton’s jelly often had more than two cytoplasmic extensions creating a stellate appearance. The density of fibroblastic cells, like collagen, was higher around the vessels than around the umbilical epithelium (e).

Magnification a-d X 1000, e X400, f X 100.

Figure 2.6: The umbilical epithelium during umbilical cord development. Up to 10 weeks (a), the umbilical epithelium consisted of a single layer of cells (red). The epithelium then became bilaminar between 10 and 20 weeks (b) and by term was as thick as 3-5 layers in certain regions (c). Variability in number of epithelial cell layers was seen throughout gestation within individual sections and between samples of similar age. In particular, at term, the epithelium could range from as many as 5 layers (c) to being bilaminar in closely adjacent regions (d).

Magnification, a-b X 1000, c-d X 400.

EP = Epithelium, WJ = Wharton's jelly.
2.4 Discussion

This study demonstrated that several developmental events can be documented histologically during normal umbilical cord development. My results defined a clear pattern of morphogenesis for the umbilical cord's growth and development. Early in gestation, the vein and arteries were structurally similar, but became increasingly distinct with the progression of gestation. There was continuous collagen deposition in the Wharton’s jelly and increasing complexity of the umbilical epithelium. These changes suggest a progressive maturation of the umbilical cord and raise some interesting hypotheses regarding the regulation of umbilical cord development.

The effects of cord contraction became increasingly pronounced during gestation. Others have observed this effect and suggest in addition to the progressive decrease in umbilical vascular length, contraction also results in the thickening of the umbilical vessels and obliteration of their lumens (50). In my study, these changes first became evident between 15 and 20 weeks and were prominent features in the term umbilical artery. Protrusion of the umbilical endothelial nuclei is another morphological feature produced by contraction (167) and could be seen as early as 12 weeks in both the arteries and vein. When the umbilical vessels are distended, vascular elastin is concentrated in sheets rather than exhibiting the dispersed, wavy pattern that I observed in the collapsed and contracted cord near term (50). Finally, the stellate shape of Wharton’s jelly fibroblasts that predominated during gestation, is also attributed to contraction (44). Thus, there are striking morphological differences between the distended and the
collapsed umbilical cord, which suggests that caution be taken when interpreting developmental changes in tissue thickness, cellular appearance, and structural orientation.

Aside from these concerns, my observations are consistent with several other developmental studies of the umbilical cord (7;10;57;167;168). In the developing umbilical vasculature, I found that the umbilical arteries and vein were structurally similar between 6 and 16 weeks. Similar observations were made by Sexton et al. (7). Sexton et al., and I also found that the umbilical artery and vein both contained a single layer of endothelial cells surrounded by vascular smooth muscle cells, with elastin first appearing between 14 and 16 weeks (7). In contrast to my work, Sexton et al., does not differentiate between arterial and venous elastin deposition and only mentions the existence of an inner region in the umbilical arteries where I observed an increase in proteoglycan staining.

The appearance and formation of an inner region within the umbilical arteries has previously been described by Monie (169). He described this region as an subendothelial cushion, and showed that these cushions are present during the major portion of fetal life. Monie also suggests structural similarities between the umbilical arterial subendothelial cushions and those seen in arteries, like the ductus arteriosus (171-173). Normally, the subendothelial cushion consists of two layers. The inner layer, subjacent to the lumen, is called the proteoglycan layer because it contains an abundance of finely reticulated nonfibrous connective tissues identified as proteoglycan ground substance. The thicker outer layer is referred to as the musculoelastic layer because of its abundance of smooth muscle cells and elastic fibers (40).
The increase in proteoglycan staining adjacent to the intima observed in the umbilical arteries between 20 weeks and term in this study confirms that a proteoglycan layer forms during umbilical arterial development. Supporting this idea is ultrastructural data showing that the umbilical smooth muscle cells in this region have a similar loose arrangement and predominant synthetic phenotype typical of cells in the proteoglycan layer (22);(40). There was also a clear demarcation of a musculoelastic region between the proteoglycan and outer smooth muscle layer by 20 weeks. Thus, both components necessary for a subendothelial cushion are present in the umbilical arteries and is a normal developmental event in the human umbilical cord.

As previously stated, the structure of the subendothelial cushion in the umbilical artery resembles that of the ductus arteriosus (169). Elastin is also fragmented in the ductus and umbilical vessels and at birth both structures must occlude their blood flow. Recent studies on cultured endothelial and smooth muscle cells derived from the sheep lamb ductus have revealed that transforming growth factor beta is an important regulator (174) of ductal cushion formation. Specifically, TGF-β is associated with the fragmentation of ductal elastin, and migrations of smooth muscle cells (28;174;175). In vivo closure of the ductus arteriosus after birth corresponds with an increase in expression of all three mammalian TGF-β isoforms (176). Thus, based on structural and functional similarities I suggest that TGF-β may control similar aspects of development in the umbilical vessels.

My observations of the Wharton’s jelly were consistent with those of Schoenberg, who showed an increase in formed collagen fibers and a tendency for these fibers to aggregate with increasing age (58). Increased Wharton’s jelly collagen deposition also
coincided with the developmental differentiation of Wharton's jelly fibroblasts (56) into cellular phenotypes attributed to myofibroblasts. TGF-βs can stimulate collagen synthesis and accumulation in cultured fibroblasts (177-180) and during wound healing, TGF-β can induce myofibroblast formation (181);(182;183). Thus, in addition to its effects on the umbilical vasculature, I suspect TGF-βs may also be intimately involved with the formation, organization and differentiation of the Wharton's jelly.

Finally, increased layering of the umbilical epithelium during development has previously been documented in both human and rat (10);(168). Ultrastructurally, in the rat, the umbilical epithelium appears to retain a morphological similarity to the amniotic epithelium during development, whereas the umbilical epithelium in humans develops into a structure that resemble the fetal epidermis (46). Interestingly, intense immunostaining of TGF-β isoforms during development of the rabbit epidermis suggests a role in regulating morphogenesis and growth (184). Thus, TGF-βs may also have an important role in regulating morphogenesis and growth in the developing umbilical epithelium.
2.5 SUMMARY

The development of the umbilical cord is complex and dynamic process. Movat’s pentachrome staining allows for the visualization of numerous developmental events which, based on structural and functional comparisons with other tissues, suggests the TGF-β isoforms may be key regulators in umbilical cord development.
CHAPTER 3:

TGF-β Expression during Normal and Pathological Umbilical Cord Development
3.1 Introduction

In the previous chapter, I described the morphological development of the umbilical cord and suggest potential roles for the TGF-β isoforms in the development of several umbilical structures. The purpose of this study was to define the time course of expression for the three mammalian TGF-β isoforms during umbilical cord development using (a) semi-quantitative western blotting for total protein and (b) immunohistochemistry and in situ hybridization for cellular localization of TGF-β protein and mRNA, respectively. This study also determined total TGF-β protein expression in umbilical cords collected from pre-eclamptic pregnancies and compared it to age matched controls.
3.2 Material and Methods

3.2.1 Sample Collection:

Sample collection, fixation and processing were performed as outlined in section 2.2.1. For pre-eclamptic umbilical cords, clinical information is summarized in table 3.1. For protein extraction, segments from the middle of normal umbilical cords were cut, flash frozen in liquid nitrogen and stored at -70°C until use. Pre-eclamptic umbilical samples were not processed for histology due to an inability to fix samples within a reasonable period of time following delivery (>3 hours). Therefore, pre-eclamptic umbilical cords samples were assessed exclusively using frozen samples for protein extraction.

3.2.2 Protein Extraction:

Protein extraction was performed using the following protocol modified from Steward et al. (143). A one gram sample of frozen umbilical cord from each fetus was pulverized under liquid nitrogen using a mortar and pestle. Then homogenized in an extraction solution, containing 1 μg/ml PMSF, 10 μg/ml Aproptin, 3.75 ml 95% Ethanol, 1.92 mL water, and 75 μL concentrated hydrochloric acid. After 1 minute of homogenization sample were placed on a shaker at 4 °C overnight. Samples were then centrifuged (Sorvall RT6000B) at 4000 rpm for 5 minutes to pellet insoluble material. The supernatant was then collected and adjusted to a pH of 5 using concentrated ammonia. Following this, 50-60 μL of 2 M ammonium acetate (pH 5.2) was added to the
sample solutions and caused a precipitate to form, which was removed by a second centrifugation at 4000 rpm for 5 minutes. Again the supernatant was collected and was combined with 4 parts (95% v/v) ethanol and 2 parts diethyl ether, mixed and allowed to precipitate at -20°C for 48 hours. The mixture was then centrifuged at 4000 rpm for 5 minutes, the supernatant poured off and the pellet allowed to air dry. The dried pellet was dissolved in 1 Molar acetic acid (200-500 µL) and dialyzed (Sigma St. Louis Mo.) overnight in beaker filled with 1 M acetic acid (~ 500 mL). Protein concentration was then determined using a standard Bradford assay (185) using Bio-Rad protein assay dye (Bio-Rad, Richmond, CA).

3.2.3 SDS-Page and Western Blotting:

Samples containing 15 µg total protein were aliquoted, diluted with 1 M acetic acid to a final volume of 15 µL and then combined with 10 µL 2X Tris-glycine native sample buffer. Samples were then boiled for 10 minutes, cooled, and loaded onto pre-made 4-20% Tris-glycine gradient gels (Novex Corp. San Diego, CA). Protein was separated by polyacrylamide gel electrophoresis according to the methods of Laemmlli (186) for 2-2.5 hours at 125 volts. Gels were then electroblotted to 0.22 µm polyvinylidene difluoride (PVDF) membrane (Immubolon-P Millipore Corp., Bedford, MA) for 1.5 hours at 300 milliamps and then allowed to air dry overnight. Before immunoblotting, the membrane was first permeabilized by immersion in methanol for 20 seconds. Methanol was then removed by two five-minute washes in Tris-buffered saline “TBST” (20 mM Tris base, 137 mM NaCl, pH 7.6 with 0.1% Tween-20) followed by blocking of non-specific binding with 5% wt/vol skim milk in TBST for one hour.
Excess block was removed by three 5-minute washes with TBST before application of the primary antibody. The polyclonal rabbit TGF-β primary antibodies (200 μg/mL Santa Cruz, CA) were diluted 1:1000 in blocking solution and allowed to incubate, on the membrane, for 1 hour at room temperature. Excess antibody was removed by one 15 minute TBST wash followed three 5 minutes TBST washes. After the washes, a goat polyclonal anti-rabbit secondary antibody was applied, which was conjugated with horseradish peroxidase (Amersham International, England). The secondary antibody was diluted 1:1000 in block and allowed to incubate on the membranes for 1 hour at room temperature. Excess antibody was again removed by TBST washes and proteins detected using the Amersham ECL detection system (Amersham International, England). Blots were then exposed to autoradiographic film (Kodak Film).

For gestational studies, a minimum of 3 samples were extracted for each age range (11-12 wk N=3, 14-16 wk N=4, 19-20 wk N=4, 25-26 wk N=3, 39-40 wk N=4) while for the pathological study, each pre-eclamptic cord had its own age matched control. Recombinant TGF-βs were included to demonstrate specificity and cross-reactivity of the antibodies. As a negative control I preabsorbed the TGF-β antibodies with isoform specific peptides (Santa Cruz, CA) and demonstrated a dramatically reduced signal detection.
3.2.4 Immunohistochemistry:

Sections were dewaxed in xylene, dehydrated in a descending ethanol series then placed in phosphate buffered saline (PBS) for 5 minutes. Sections were then incubated for 30 minutes in a 5% hydrogen peroxide methanol solution to quench endogenous peroxidase activity, followed by three 5 minute washes in PBS. Antigen unmasking used 0.125% trypsin in PBS for 15 minutes at 37°C since it produced more consistent results than pepsin digestion and did not cause samples loss like the sodium citrate microwave method. Excess enzyme was removed by 3 5 minute PBS washes and was followed by blocking of non-specific binding using 1% bovine serum albumin (BSA), 1% normal horse serum (NHS) and 4% normal goat serum (NGS), in PBS. The blocking solution was replaced, after one hour, by one of the three TGF-β primary antibodies used for our western blotting. The primary antibodies were diluted 1:500 in blocking solution, and the sections were incubated at room temperature for 2 hours. Excess antibody was removed by three 5 minute PBS washes followed by a two hour incubation of samples with a horse anti-rabbit biotinylated secondary antibody (Vetastain Universal, Vector, CA.) diluted 1:1000 in blocking solution. Sections were washed three times for 5 minutes in PBS then allowed to incubate with the avidin biotin peroxidase complex (ABC) (Vectastain elite, Vector, CA.) for one hour at room temperature. Using Diamionobenzidine 3',3',4',4' tetraminobiphenyl (DAB), a positive signal was indicated by a brown precipitate following a 5 minute incubation in the dark. Samples were then rinsed in distilled water, counterstained for 30 seconds with Meyer's hematoxylin, dehydrated through an ascending alcohol series, cleared in xylene and mounted with permount.
A minimum of 4 different cords samples were tested within each age group to minimize variation due to differences in fixation and processing. Individual experiments always contained at least two samples from each age group and often contained a 6-week human placenta sample as a positive control, or a section was run without primary antibody as a control for non-specific binding. Periodically, antibody specificity was also tested by preabsorption of the antibody with a specific peptide (negative control).

3.2.5 Indirect In Situ Hybridization using DIG labeled mRNA probes:

TGF-β3 riboprobes were generated using either the T7 or Sp6 polymerase by linearizing a pCR®II vector (Hind III for antisense, XbaI for sense) for transcription of a 304 base cDNA fragment complementary to TGF-β3 (187). During transcription, probes were labeled with dUTP-digoxigenin by the random primer method as described in the RNA labeling and detection kits from Boehringer Mannheim (Laval, Quebec, Canada). In situ hybridization was then conducted according to the following protocol modified from Braissant and Wahli, (188). Under RNAse free conditions sections were dewaxed in xylene, rehydrated through a descending ethanol series, then post-fixed in 4% paraformaldehyde (PFA) for 10 minutes to help maintain tissue morphology. Tissue sections were then washed three times for 5 minutes in DEPC treated PBS. Tissue permeabilization utilized enzymatic digestion with 10 μg/mL proteinase K in buffer for 20 minutes at 37°C and was preferred over the sodium citrate microwave method, since it did not cause tissue folding. After tissue permeabilizing, excess enzyme was removed by three 5 minute DEPC-PBS washes and proceeded a second post-fixation in 4% PFA of 5 minutes. Sections were then washed in a 0.1% active DEPC/PBS solution twice for 5
minutes to remove excess PFA and destroy endogenous RNAse. After the tissue sections were washed they were then prehybridized for 2 hours at 50°C in a solution containing 5X SSC, 50% formamide and ssDNA. The prehybridization solution was replaced with the hybridization solution containing 5X SSC, 50% formamide, ssDNA and the riboprobe diluted 1:100. Tissue sections were then incubated overnight at 50°C. Excess probe was removed by washes in 2X SSC for 30 minutes at room temperature, followed by sequential washes in 2X SSC and 0.1X SSC for 1 hour at 50°C. Before application of anti-DIG antibody, endogenous alkaline phosphatase activity was blocked with 0.02 M levimasole in maleic acid for 15 minutes. The monoclonal alkaline phosphatase conjugated Anti-DIG antibody (Boehringer Mannheim, Que) was diluted in a 1 X maleic acid blocking solution and allowed to incubate on the sections for 2 hours at room temperature. Excess antibody was removed by three 5 minute washes in maleic acid, followed by a 5 minute equilibration in detection buffer. Detection employed the nitro-blue tetrazolium/ 5-bromo-4-chloro-3-indolyphosphate substrate (NBT/BCIP) and was stopped once a bluish/ purple precipitate could be identified. Sections were then washed in 95% ethanol, counter-stained in methyl green for 5 minutes, dehydrated in an ascending alcohol series, cleared in xylene and mounted with permount.

A minimum of 3 different cords samples were tested for each age group to minimize variations due to fixation and processing. Individual experiments always contained at least 2 samples from each age group and contained either a placenta from molar pregnancies as a positive control, or a sample were the sense riboprobe was used as a negative control.
3.2.6 Statistical analysis:

Autoradiographic films from western blot were scanned at 200 dpi, saved as TIFF images and analyzed using ScionImage software (Scion Corp. MD.) to obtain optical density. The relative optical densities for gestational age studies were calculated based on the term (35-40 wk.) age group equaling one. For each pre-eclamptic sample and its age matched control, the total optical density of the two samples was determined. Each sample was then expressed as a fraction of the total optical density of the pair. This process was repeated for all five pairs to determine the relative optical density ratio. All data are presented as means ± the standard deviations. Statistical significant changes in TGF-β3 protein with gestational age were determined using one-way ANOVA followed by a Student Newman-Keuls multiple comparison test using sigma stat software. Differences between pre-eclamptic and age-matched controls were tested using a Student’s T-test. A p<0.05 was considered statistically significant.
3.3 RESULTS

3.3.1 Total TGF-β Protein Expression during Gestation:

TGF-β1 protein expression could not be identified by Western blot analysis at any gestational age (not shown). TGF-β3 protein expression, on the other hand was undetected in samples from 10 weeks gestation or earlier (not shown), but rose to detectable levels by 12 weeks and had significantly increased from this level by 20 weeks. TGF-β3 protein continued to rise and by term was significantly higher than at 20 weeks (figure 3.1). The TGF-β2 antibody cross-reacted with recombinant TGF-β3 (not shown) and was therefore not used for immunohistochemistry.

3.3.2 TGF-β Protein and mRNA Cellular Localization during Gestation:

Endothelium:

Throughout gestation, immunoreactivity (brown) to TGF-β1 (figure 3.2 a,d) and TGF-β3 protein (figure 3.3) could be identified in both the umbilical arterial and venous endothelium (figure 3.4). Between 8 weeks and 15 weeks, TGF-β3 mRNA staining intensity (purple) rose in the endothelium and from 15 week onwards, both protein and mRNA continued to be expressed in the arterial and venous endothelium (figure 3.5). Technical difficulties prevented the generation of a suitable TGF-β1 and TGF-β2 riboprobes.
Vascular Wall:

TGF-β1 protein immunoreactivity (brown) in the vascular wall was either low or undetectable throughout gestation (figure 3.2 a,b). TGF-β3 protein immunoreactivity, on the other hand, became stronger in the vascular wall between 8-20 weeks (figure 3.4 a-f). During this age range TGF-β3 immunoreactivity was primarily intracellular and appeared uniformly distributed throughout the smooth muscle cell layers in both the arteries and vein. Between 20 weeks and term, immunoreactivity to TGF-β3 in both the arterial and venous walls changed, from a ubiquitous to regional staining pattern. That is in certain regions TGF-β3 immunoreactivity appeared maintained (double headed arrow figure 3.3 g); however, in the bulk of the vascular wall TGF-β3 was no longer uniformly expressed.

TGF-β3 mRNA staining intensity (purple), like protein, initially increased between 8-15 weeks (figure 3.5). Individual immunopositive vascular smooth muscle cells could be seen throughout the arterial and venous vasculature at 15 weeks (figure 3.5 c, d respectively), and showed a relatively even distribution pattern. However, unlike TGF-β3 protein expression, TGF-β3 mRNA staining intensity was lower in both the arterial and venous vascular smooth muscle between 15 and 20 weeks (figure 3.5 e, f). After 20 weeks, TGF-β3 mRNA staining intensity diminished further in the umbilical vascular smooth muscle (figure 3.5 g) and was undetected at term.
**Wharton’s Jelly:**

At 8 weeks, TGF-β3 protein and mRNA (figure 3.6) were undetectable in the Wharton’s jelly fibroblasts. By 15 weeks, TGF-β3 protein, immunoreactivity was elevated in the majority of Wharton’s jelly fibroblasts cells (figure 3.6 c, d), and was maintained at 20 weeks (figure 3.5 e, f) and term (figure 3.5 g, h). TGF-β3 mRNA staining intensity (blue/purple) also rose noticeably between 8 and 15 weeks and was then maintained until term. In contrast, TGF-β1 immunoreactivity was low throughout gestation and was only identified intermittently in fibroblast cells at term (figure 3.2 c).

**Umbilical Epithelium:**

TGF-β1 protein immunoreactivity was detected in the umbilical epithelium (figure 3.2 d) throughout gestation as were both TGF-β3 protein (figure 3.7 a,c,e,g) and mRNA (figure 3.7 b,d,f,h). Variability in protein immunoreactivity and mRNA staining within sections and between samples of similar ages made it difficult to determine whether TGF-β protein or mRNA changed markedly during gestation.

3.3.3 Total TGF-Beta 3 Protein Expression from Pre-eclamptic Pregnancies.

Total TGF-β3 protein was significantly lower in umbilical cords samples from pre-eclamptic pregnancies than age-matched controls (figure 3.8).
3.3.4 Positive and Negative Controls for TGF-β3 protein and mRNA Expression.

The TGF-β3 protein antibody recognized recombinant TGF-β3 protein at a similar molecular weight (25 kDa) as in our protein samples and did not cross-react with recombinant TGF-β1 (figure 3.9 a and b respectively). Immunoreactivity to TGF-β3 protein, within a 6 week human placenta, was accurately expressed in the trophoblast and stromal cells as seen by Vuckovic et al., (189) (figure 3.9 c). Preabsorption of the TGF-β3 antibody with a synthetic TGF-β3 peptide showed very little non-specific binding (figure 3.9 d). Immunoreactivity to TGF-β3 mRNA within placentas from molar pregnancies was similar to that seen by Caniggia et al., (190) (figure 3.9 e). Very little non-specific binding was seen when samples were incubated with a sense, rather than antisense, riboprobe (figure 3.9 f).
### TABLE 3.1: Clinical Data on Pre-eclamptic Umbilical Cord Samples

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gestational age at Delivery (weeks)</th>
<th>Maximum Arterial Blood Pressure (mmHg) systolic/ Diastolic</th>
<th>Proteinuria</th>
<th>Umbilical Artery Doppler</th>
<th>Sex of Fetus</th>
<th>Birth Weight (grams)</th>
<th>&lt;10th percentile birth weight for gestational age</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>26</td>
<td>220/120</td>
<td>3+</td>
<td>AEDF</td>
<td>F</td>
<td>760</td>
<td>Yes</td>
</tr>
<tr>
<td>B</td>
<td>26</td>
<td>170/110</td>
<td>3+</td>
<td>AEDF</td>
<td>M</td>
<td>810</td>
<td>No</td>
</tr>
<tr>
<td>C</td>
<td>27</td>
<td>150/95</td>
<td>1+</td>
<td>AEDF</td>
<td>M</td>
<td>555</td>
<td>Yes</td>
</tr>
<tr>
<td>D</td>
<td>35</td>
<td>140/90</td>
<td>1+</td>
<td>Normal</td>
<td>M</td>
<td>1510</td>
<td>Yes</td>
</tr>
<tr>
<td>E</td>
<td>37</td>
<td>180/100</td>
<td>1+</td>
<td>Normal</td>
<td>M</td>
<td>2840</td>
<td>No</td>
</tr>
</tbody>
</table>

(AEDF: Absent end diastolic blood flow velocity.)
Figure 3.1: In the upper panel (a), a sample blot (two sample at each age group) illustrates that total TGF-β3 protein expression increases during human umbilical cord development. The lower panel (b) is the developmental profile of TGF-β3 protein where data is expressed as relative optical density (mean ± SD; n = 3). Bars with different letters are significantly different from each other (p< 0.05).
a) $\leftarrow 25 \text{kD}$

$12\text{wk}$  $15\text{wk}$  $20\text{wk}$  $25\text{wk}$  Term ($35-40\text{wk}$)

b)

![Bar chart showing relative optical density with gestational age: $12\text{wk}$, $15\text{wk}$, $20\text{wk}$, $25\text{wk}$, and Term ($35-40\text{wk}$).]
**Figure 3.2:** TGF-β1 protein immunoreactivity (brown) in the umbilical cord during gestation. Within the endothelium, TGF-β1 could be found throughout gestation (a,b). TGF-β1 immunoreactivity was not found in the umbilical vascular smooth muscle cells (b) during gestation, and was only found intermittently in Wharton’s jelly fibroblasts (c). Like the endothelium, TGF-β1 immunoreactivity was found throughout gestation in the umbilical epithelium (d).
Figure 3.3: TGF-β3 protein immunoreactivity (brown) in the umbilical endothelium and inner smooth muscle layers during gestation. For both the artery (a,c,e,g) and vein (b,d,f,h), TGF-β3 immunoreactivity could be seen in individual endothelial cells throughout gestation. In the vascular smooth muscle, TGF-β3 immunoreactivity increased between 8 and 20 weeks for both artery and vein, and then decreased towards term.

Magnification 1000X.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s Jelly.
Figure 3.4: TGF-β3 protein immunoreactivity (brown) in the umbilical vasculature during gestation. Between 8 and 20 weeks TGF-β3 immunoreactivity increased in both the artery (a,c,e) and vein (b,d,f). At term TGF-β3 immunoreactivity becomes regionally specific (g,h). In certain regions TGF-β3 immunoreactivity remains strong (double headed arrow), but throughout the majority of vessel staining became patchy.

Magnification 8 wk = 1000X, 15 wk = 400X, 20wk = 200X, Term = 100X.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s Jelly
Figure 3.5: TGF-β3 mRNA staining (blue/purple) in the umbilical vasculature during gestation. For both the artery (a,c,e,g) and vein (b,d,f,h) TGF-β3 mRNA could be seen in endothelial cells throughout gestation. In the vascular smooth muscle cells TGF-β3 mRNA increased between 8 and 15 weeks, declined at 20 weeks and was undetected at term.

Magnification 8 wk = 1000X, 15 wk = 400X, 20wk = 200X, Term = 100X.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s Jelly
Figure 3.6: TGF-β3 protein (a,c,e,g) and mRNA (b,d,f,h) in Wharton's jelly fibroblasts during gestation. At 8 weeks neither protein or mRNA could be detected in the fibroblasts, but between 8-15 weeks protein and mRNA immunoreactivity rose and were maintained until term.

Magnification 1000X.

WJ = Wharton’s jelly
**Figure 3.7:** TGF-β3 protein (a,c,e,g) and mRNA (b,d,f,h) in the umbilical epithelium during gestation. Throughout gestation both protein and mRNA could be identified in the umbilical epithelium.

Magnification 1000X.

WJ = Wharton's Jelly, EP = Epithelium
Figure 3.8: TGF-β3 total protein expression in normal and pre-eclamptic umbilical cords
Panel (a) is a sample western blot of all five pre-eclamptic umbilical cords (capital letter A-E) and age matched controls (lower case c's) and illustrates that at each age group TGF-β3 is lower in the pre-eclamptic samples. The lower panel (b) is the data comparing TGF-β3 protein expression in normal and pre-eclamptic umbilical cords expressed as a relative optical density ratio (mean ± SD; n = 5). Bars with different letters are significantly different from each other (p< 0.05).
a) ← 26 wk → 27wk 35wk 37wk

25 kDa

c A c B c C c D c E

b)

Relative Optical Density Ratio

0.8

0.7

0.6

0.5

0.4

0.3

0.2

0.1

0

Normal

Pre-eclamptic
Figure 3.9: Experimental controls for TGF-β3 Western blotting (a,b), protein staining (c,d) and in situ hybridization (e,f). Recombinant TGF-β3 (r TGF-β3) protein migrated at the same level as the protein detected in the human cords by the TGF-β3 specific antibody (a). The TGF-β3 antibody showed very little cross reactivity with recombinant TGF-β1 (r TGF-β1) (b). TGF-β3 protein immunoreactivity localised to the trophoblast and stromal cells of a 6 week human placenta (c), and showed very little non-specific binding in the term umbilical artery when preabsorbed with a specific TGF-β3 peptide (d). The antisense TGF-β3 riboprobe specifically hybridized to the trophoblasts from molar placentas (e), while the sense TGF-β3 riboprobe showed very little hybridization in the term umbilical artery (f).

Magnification 6 wk placenta =1000X, Molar Placenta =400X, Term Umbilical artery =200X. L = Lumen, SM = Smooth Muscle, T = Trophoblast, M = Mesenchyme, EVS = Extravillous Space.
a) rTGF-Beta 3  Term Cord

b) rTGF-Beta 1  Term Cord

c) 6 wk Placenta

d) Term umbilical artery

e) Molar Placenta

f) Term umbilical artery
3.4 Discussion

In this study, I demonstrated that during umbilical cord development TGF-β3 has a distinct and overlapping expression pattern compared with TGF-β1 and was significantly decreased in umbilical cords from pre-eclamptic pregnancies. Under non-reducing conditions, umbilical cord TGF-β3 migrated on SDS-PAGE with identical mobility as recombinant TGF-β3 and was selectively recognized by a TGF-β3 specific antibody by Western blot analysis. Presently, the only other study to identify a change in arterial TGF-β expression during gestation, by Western blotting, found that TGF-β1 expression decreased in ductus arteriosus endothelial cells with increasing age (191). However, because they examined TGF-β1 rather than TGF-β3 and because they employed cell culture to obtain the protein quantities necessary for western blot analysis, my results are not directly comparable.

I identified cell specific changes in TGF-β3 protein immunoreactivity during gestation that were in accord with our Western blot results, between 8 and 20 weeks. In endothelium, and the epithelium, both TGF-β3 and TGF-β1 protein were identified throughout gestation, whereas in the Wharton’s jelly fibroblast cells TGF-β3 immunoreactivity increased between 8 and 15 weeks. Within the vascular wall TGF-β1 immunoreactivity was low throughout gestation, while TGF-β3 immunostaining was perceptible on an individual cell basis after 10 weeks, and showed an overall increase. Currently no other study has investigated changes in TGF-β3 protein immunoreactivity in human fetal tissues at different during gestational ages. However, in mouse lung vessels, TGF-β3 immunoreactivity is higher in smooth muscle cells of blood vessels with thicker
smooth muscle sheaths than those with thinner walls (192). Thus the increase in umbilical vascular TGF-β3 immunoreactivity, between 10 and 20 weeks is likely due to the increase in number of condensed vascular smooth muscle layers.

In the term umbilical cord, comparison of TGF-β3 protein immunostaining between my data and that of Stewart et al., (143) revealed several discrepancies. Both studies utilized formalin immersion fixation of whole cord samples and found positive immunostaining in the epithelial cells surrounding the cord. However, in contrast to our data, Stewart et al., found only moderate staining for TGF-β3 in the Wharton’s jelly fibroblasts and did not detect TGF-β3 in the endothelial cells. Stewart’s data also suggests the vascular smooth muscle cells are the major cell type, in the cord, immunostaining for TGF-β3 at term. In contrast, at this gestation we identify a patchy TGF-β3 staining pattern in the vascular walls, where numerous smooth muscle cells show low TGF-β3 immunoreactivity. The reason for these inconsistencies may be partially explained by two specific deviations in staining methods. First, I used a trypsin incubation to assist antibody infiltration in our formalin fixed specimens, where Stewart et al., did not. This difference may explain why Stewart et al., saw TGF-β3 immunoreactivity in the endothelial cells of their frozen sections, but not formalin fixed. Second, the concentration at which Stewart et al., applied their primary antibody (15-30 μg/mL) was notably higher than that applied on my samples (0.4 μg/mL). Although the antibodies were from different companies when I used lower primary antibody dilutions (1:100) for my antibody I found TGF-β staining everywhere in the term cords regardless of isoform. At these higher concentrations, the gestational changes I observed for TGF-β3 were also abolished. Thus, it is my opinion that the TGF-β3 immunolocalization
pattern I identified is more representative of the true pattern of TGF-β3 expression in the umbilical cord.

TGF-β3 mRNA colocalized with TGF-β3 protein in the endothelium, epithelium, and Wharton’s jelly fibroblasts throughout gestation. In both endothelium and epithelium, protein and mRNA are expressed throughout gestation, while in the Wharton’s jelly neither protein or mRNA could be identified at 8 weeks, but were increased by 15 weeks and remained elevated until term. During mouse embryogenesis, the fact that TGF-β3 mRNA is also found in the fibroblasts of the umbilical cord and in intima of certain major blood vessels (193) supports our observations and suggests that TGF-β3 may act in an autocrine fashion in these cells.

Within the vascular smooth muscle cells of the umbilical artery and vein, the expression profiles of TGF-β3 protein and mRNA did not colocalize throughout gestation. Between 8 and 15 weeks, an increase in TGF-β3 mRNA corresponded with increased protein immunoreactivity in the vascular smooth muscle cells for both the artery and vein. However, from 15 to 20 weeks TGF-β3 mRNA staining intensity decreased in the vascular smooth muscle cells and, by term, was not seen in appreciable amounts in either artery or vein even though TGF-β3 protein was still found. This change suggests that umbilical smooth muscle cells at term do not have the ability to produce their own TGF-β3. Potential sources for TGF-β3 in the smooth muscle cells at term include paracrine production from either the umbilical endothelium, Wharton's jelly fibroblasts or even the umbilical epithelium. In addition, TGF-β3 may come from a latent reservoir existing in the extracellular matrix, which because it is in a latent state was not detected by the antibody.
The final part of my study provides the first evidence of altered growth factor expression in pre-eclamptic umbilical cords. Caniggia et al., (190), using placental explants, first demonstrated that TGF-β3 may be a clinically important molecule in pre-eclampsia. They showed that pre-eclamptic placentae, between 27 and 34 weeks of gestation, overexpress TGF-β3 and exhibit a hypoinvasive phenotype in vitro. They also demonstrated that trophoblast differentiation towards an invasive phenotype can be restored by disrupting TGF-β3 activity and synthesis. Although my study indicates that TGF-β3 is underexpressed in pre-eclamptic umbilical cords, between 26 and 37 weeks of gestation, both studies suggest that TGF-β3 may participate in the pathogenesis of pre-eclampsia.
3.5 Summary

In summary, I have demonstrated the time course for changes in TGF-β isoform expression during umbilical cord development. This study indicates that TGF-β3 is gestationally regulated and may be important in pre-eclampsia. To my knowledge this is the first study to combine semi-quantitative changes in total TGF-β protein with protein and mRNA localization for the investigation of TGF-βs in human development. It is also the first study to provide a detailed account for changes in TGF-β protein and mRNA localization within a developing vascular tissue. Considered together, these data, along with TGF-βs multifunctional nature, indicate that endogenously produced TGF-βs play an important role in the normal physiologic development of the human umbilical cord.
CHAPTER 4:

General Discussion
4.1 Introduction

Results from the studies in this thesis demonstrate that the developing human umbilical cord undergoes numerous morphological changes and displays cell specific changes in expression of the local regulator, TGF-β3. A significant decrease in total TGF-β3 expression was also associated with umbilical cords affected by pre-eclampsia. In the following section, I will compare my morphological and TGF-β data in order to generate hypotheses regarding TGF-β3’s function and regulation in umbilical cord development.

4.2 Cell Specific Changes In Morphology and TGF-β3 Expression during Umbilical Cord Development

Endothelium: Morphologically, umbilical endothelial cell nuclei increasingly protruded into the vessel lumen during development, however this was most likely a contractile artifact. Consequently, speculations on TGF-β3 function in the umbilical endothelium are difficult to draw. In vitro, TGF-β3 and TGF-β1 both inhibit proliferation and migration as well as promoting neovascular processes in endothelial cells from both large and small vessels (194). Therefore, since TGF-β1 and TGF-β3 are both found in the umbilical endothelium throughout gestation, I hypothesize that TGF-β1 and TGF-β3 have overlapping functions in the umbilical endothelium. Whether umbilical endothelial TGF-βs increase hyaluronic acid synthesis, like TGF-β1 does in culture endothelial cells from the ductus arteriosus (195), or regulate vascular reactivity through the inhibition of nitric oxide synthase (196;197) or stimulation of endothelin (198;199) remains to be established.
In chapter 1 section 5.2, I introduced the idea that TGF-β expression is influenced by shear stress. In adult vessels increases in shear stress result in an endothelium mediated increase in vascular luminal diameter (74). However, due to the progressive contraction of umbilical vessels with increasing gestation, changes in luminal diameter could not be measured. As a result, although both TGF-β1 and TGF-β3 are found throughout gestation in the umbilical endothelium, a relationship between TGF-βs and shear stress in the umbilical cord could not be defined.

*Vascular Wall:* Unlike the endothelium TGF-β1 and TGF-β3 do not colocalize in the umbilical vascular wall, which suggests TGF-β3 has an isoform specific in umbilical vascular wall morphogenesis. One of the most striking morphological changes, in the developing umbilical vasculature, was the formation of the arterial subendothelial cushion. Cushion formation occurred between 20 weeks and term and coincided with the most conspicuous changes in vascular smooth muscle cell TGF-β3 protein and mRNA expression. A specific correlation between TGF-β expression and vascular wall morphological changes could not be clearly defined. However, the fact that TGF-β3 inhibits the proliferation of cultured vascular smooth muscle cells, while also stimulating their migration (194) suggests TGF-β3 may regulate specific aspects of umbilical smooth muscle maturation. Consistent with this idea is the fact that, between 8 and 20 weeks, increased expression of TGF-β3 in the vascular wall coincides with a progressive increase in vascular smooth muscle myofilament content and ratio of contractile to synthetic phenotypes during gestation (7). While the decrease in TGF-β3 vascular smooth muscle mRNA expression I observed, between 20 weeks and term, is similar to that seen for TGF-β2 transcripts during the later phases of rabbit aortic arch maturation
Thus I hypothesize that TGF-β3 promotes the migration of cells to the umbilical vascular walls, in early gestation, where they can differentiate into contractile smooth muscles cells. Once these cells differentiate, TGF-β3 expression then decreases in the vascular wall, and allows for the formation of secondary vascular structures, like the subendothelial cushion in the umbilical arteries.

Whether the induction of umbilical smooth muscle migration and differentiation is a direct or indirect effect of TGF-β3 remains to be established. TGF-βs can directly influence vascular smooth muscle differentiation through their effects on cellular proliferation (201;202), contractile protein synthesis (203), and their ability to reorganize the cytoskeleton (204). TGF-βs ability to influence vascular smooth muscle collagen (205;206;207), elastin (208) and integrin (209) expression also suggests that TGF-βs may influence umbilical vascular smooth muscle phenotype indirectly through alteration in matrix composition (210).

In chapter 1 section 5.2, in addition to the idea that shear stress influences TGF-βs, TGF-βs are also influenced by changes in medial wall tension. In adult vessels, chronic increases in wall tension are associated with increases in vascular wall thickness (74). In my studies the effects of changes in medial wall tension on umbilical TGF-β isoform expressions were not assessed because the umbilical vessels became increasingly contracted with age. This caused changes in vascular wall thickness to become overexaggerated with gestation and altered vessel radius.

*Wharton's jelly:* In the Wharton's jelly a potential correlation exists between collagen deposition and TGF-β3 expression. At 8 weeks, when very little collagen could be seen, neither TGF-β3 protein nor mRNA could be identified, yet by 15 weeks when
collagen was increasing, TGF-β3 mRNA and protein expression had become broadly expressed. From 15 weeks onwards, collagen continued to increase in the Wharton's jelly while the fibroblasts maintained their TGF-β3 expression. I hypothesize that TGF-β3 promotes the production and accumulation of collagen by Wharton's jelly during development, in a manner consistent with that by which TGF-β3 acts as a potent stimulator of procollagen metabolism in cultured fibroblasts (211).

_Umbilical Epithelium:_ Like the umbilical endothelium, the umbilical epithelium consistently showed both TGF-β1 and TGF-β3 expression throughout gestation. While morphologically I identified changes that were consistent with the idea that the umbilical epithelium develops in a pattern similar to that of the fetal epidermis (46). In the adult epidermis TGF-β3 co-localizes with TGF-β1 (212), however, currently TGF-β3 expression has not been characterized in the fetal epidermis. TGF-β1 immunoreactivity, on the other hand, is intense during rabbit skin development (184) and appears to be important in regulating epithelial morphogenesis. While in the mouse the induction epidermal hyperplasia is associated with significant down-modulation of all three TGF-β isoforms (213). Therefore, based on the co-localization of TGF-β1 and TGF-β3 during umbilical epithelial development, I hypothesize that both TGF-β isoforms are important in regulating umbilical epithelial morphogenesis and are necessary tissue homeostasis.

4.3 **Consequences of Decreased TGF-β3 in Pre-eclamptic Umbilical Cords**
The significant decrease in total TGF-β3 expression I saw in pre-eclamptic umbilical cords may contribute to several of the structural, morphological and functional changes associated with pre-eclamptic umbilical cords. Specifically, there is an abnormal deendothelialization in pre-eclamptic umbilical arteries (214), which is associated decrease vascular contractility to serotonin (215) and deviations in fetal blood vasoactive compounds including reduced protacyclin and nitric oxide synthase activity in the cord, combined with increased endothelin (216-221). In the vascular wall of pre-eclamptic umbilical arteries, vascular smooth muscle cells show morphological features of metabolic activation and decreased active tension (91), combined with a doubling of collagen (222), a reduction in elastin (27) and a decreased hyaluronic acid content (89). Together these alterations suggest that of the pre-eclamptic umbilical vessel maturation maybe altered. Since I hypothesized that umbilical TGF-β3 is involved in umbilical vascular smooth muscle migration and differentiation I would anticipate that TGF-β3 is altered in pre-eclamptic vessels. Therefore, since TGF-β3 is significantly decreased in whole cord extracts from pre-eclamptic cord I hypothesize that TGF-β3 is reduced in pre-eclamptic umbilical vessels. In the Wharton’s jelly, quantitatively, there was no change in collagen content in pre-eclamptic cords (41), which suggests that fibroblast TGF-β3 expression would be unchanged in pre-eclampsia. Whereas, in the umbilical epithelium, no study has examined cellular or extracellular changes associated with pre-eclampsia, so I cannot speculate on whether TGF-β3 is altered at this site.

Interestingly pre-eclamptic umbilical cord is also associated with reduced estrogen (87);(88) and elevated cortisol levels (85;86) in the fetal blood. In chapter 1 section 5.2 I stated that hormones have the ability to influence TGF-βs expression, in
which estrogen specifically stimulates TGF-β3 (165) and cortisol suppresses it (166).

Consequently, the decrease in pre-eclamptic umbilical TGF-β3 may be partially the result of decreased estrogen levels and increased cortisol levels.
4.4 Future Studies:

Considerable insight into TGF-β isoform expression during umbilical cord development was gained by the experiments in this thesis. Results from these studies showed that TGF-β3 may be associated with several of the normal developmental changes seen throughout the umbilical cord and could be a key factor in several abnormalities associated with pre-eclamptic umbilical arteries. These findings lead to additional questions regarding the exact physiologic role of TGF-βs in umbilical cord, which is the focus of the following section.

(1) Histological Immunolocalization of TGF-β3 in Pre-eclamptic Umbilical cords.

Results from this thesis show that total TGF-β3 expression is decreased in pre-eclamptic umbilical cords. Currently it is not known whether this decrease is a global reduction throughout the entire umbilical cord or whether it is regionally specific. Thus, a study examining the localization of TGF-β3 protein and mRNA in pre-eclamptic umbilical cords compared to age matched controls would further enhance our understanding of the potential functions of TGF-β3 in umbilical cord development.

(2) TGF-β Receptor expressions in Normal and Pre-eclamptic umbilical cords.

TGF-β isoforms must bind to membrane bound receptors to elicit a biological response in which the Type I and type II receptors are necessary to initiate signal transduction. A preliminary study showed that the type I and type II receptors are highly expressed throughout normal umbilical cord development (appendix A). Future studies should further define the expression patterns for TGF-β receptors in the umbilical cord.
during normal development and compare these data with that obtained in pre-eclamptic umbilical cords. In doing so we can better ascertain where and when TGF-βs elicits a response in developing umbilical cord.

(3) TGF-βs influence on umbilical cord Proliferation.

In vitro TGF-β1 and TGF-β3 both reduce endothelial and smooth muscle proliferation (194). In vivo these responses appear to be influenced by factors like cell origin, differentiation state, and the surrounding extracellular matrix (223). Preliminary results during normal umbilical cord development suggest that umbilical cord proliferation decreases with increasing age (appendix B). It would be interesting to determine whether there is a correlation between TGF-β expression and umbilical cord proliferation.

(4) TGF-βs influence on umbilical cord matrix remodeling.

In my morphological data, gestational increases in collagen, elastin and proteoglycan staining were observed, and are likely due to increased synthesis and deposition, which TGF-βs can significantly influence (156). Aside from deposition TGF-βs also have the ability to influence extracellular matrix degradation (224;225). Currently, the effects of TGF-β3 on matrix degradation have not been assessed. Preliminary data shows the umbilical cord expresses several of the matrix metalloproteinases and their tissue inhibitors in distinct gestational patterns (appendix C). Future studies should further define the matrix remodeling system in the umbilical cord and assess the influence that different TGF-βs have on this system.
(5) Cell specific In vitro responses to TGF-βs in normal umbilical cords.

Since the studies in this thesis did not address the cause-effect relationship between TGF-β isoforms and specific umbilical cell types, future studies should consider this. The first step in understanding the cause-effect relationship for TGF-βs in umbilical cord development would be to establish an in vitro model that would mimic in vivo developmental changes I described. The simplest model would entail culturing primary umbilical cells (i.e. endothelial, vascular smooth muscle, fibroblast and epithelium) from different gestations and determining their TGF-β isoform expression. A valid model would show consistent levels of TGF-β1 and TGF-β3 in both the endothelium and epithelium regardless of age, exclusive expression of TGF-β3 in the vascular smooth muscle with levels peaking between 15 and 20 weeks, and an increase in Wharton's jelly fibroblast TGF-β3 expression after 8 weeks.

The successful validation of an in vitro model for TGF-β3 expression in the umbilical cord would allow studies to manipulate the in vitro environment. For instance, the effects of adding exogenous TGF-βs to specific cell types could be determined in vitro and extrapolated to the in vivo situation. Along with exogenous addition of TGF-βs, the effect of TGF-β inhibition could also be investigated.
APPENDIX A:

Expression of TGF-β receptors in the Umbilical Cord
Appendix A

For TGF-β isoforms to exert a biological response on a cell, the cell must express both the type I and type II receptors. Using protein immunohistochemistry I determined the expression pattern of the TGF-β type I receptor (figure A.1) and the TGF-β type II (figure A.2) at various gestational ages. Preliminary results indicated that both receptors were widely expressed throughout the umbilical cord during gestation. Neither western blotting nor in situ hybridization were performed to confirm these results.
**Figure A.1:** TGF-β type I receptor expression in the umbilical artery (a-d) and Wharton's jelly during gestation (e, f). Between 8 weeks and term TGF-β type I receptor immunoreactivity was highly expressed in umbilical artery and in the fibroblasts of the Wharton’s jelly.

Magnification of Vessel; 8 wk = 1000 X, 15 wk = 400 X, 20 wk = 200 X, Term = 100 X.

Wharton’s Jelly; 8 wk and Term = 1000 X.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s jelly.
Figure A.2: TGF-β type II receptor expression in the umbilical artery (a-d) and Wharton’s jelly during gestation (e, f). Between 8 weeks and term TGF-β type II receptor immunoreactivity was highly expressed in umbilical artery and in the fibroblasts of the Wharton’s jelly.

Magnification of Vessel; 8 wk = 1000 X, 15 wk = 400 X, 20 wk = 200 X, Term = 100 X.

Wharton’s Jelly; 8 wk and Term = 1000 X.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s jelly.
APPENDIX B:

Proliferation in the Umbilical Cord
Appendix B

To determine whether umbilical cord TGF-β isoform expression correlated with gestational changes in proliferation I stained histological sections at different gestations for the nuclear envelope specific epitope M1B1, which is expressed during most of the cell cycle. The staining procedure was identical to that for TGF-β3 except an additional antigen retrieval was employed prior to trypsinization involving the boiling of sections in 10 mM citrate buffer for 5 minutes. Preliminary results indicated a gestational decrease in immunopositive proliferating cells in the vasculature (figure B.1.) and amniotic epithelium, while in the Wharton’s jelly low levels of proliferation were maintained throughout gestation (not shown). Variability within sections between experiments and within similar age groups prevented comparisons with our TGF-β and morphological results.
Figure B.1: Proliferation in the umbilical arterial vasculature during gestation.

Throughout gestation, the frequency of proliferating cells (arrows indicate individual proliferating cells in b, d, f) seen in the umbilical artery decreased. The left side of the figure shows representative vessels at low magnification, and on the right side, boxed regions are enlarged. A 9 week human placenta was used as a positive control showing high proliferation in invading columns (g, h).

Magnification a, c, e, g. = 100 X, b, d, f, h. = 400 X

L = Lumen, SM = Smooth Muscle, CL = Column
APPENDIX C:

Expression of Matrix Metalloproteinases and their Inhibitors in the Umbilical Cord
Appendix C

Since TGF-βs can influence the expression and activity of the Matrix Metalloproteinases (226;227) (225), I investigated the expression of 4 MMPs and 2 of their tissue inhibitors TIMP-1 and TIMP-2 at 15, 20 and 40 weeks. Experimental methods employed were immunohistochemistry, western blotting and zymography. Preliminary results indicated that each MMP and TIMP showed a distinct gestational expression pattern. MMP-1 showed a dramatic increase in total protein expression in the third trimester (figure C.1), which coincided with a change from intracellular to extracellular MMP-1 immunoreactivity in vascular smooth muscle between 20 weeks and term (figure C.2). Total and active MMP-2 protein expression (64 kD) peaked between 15 and 20 weeks gestation (figure C.3), while MMP-2 was expressed exclusively in the fibroblasts of the Wharton’s jelly (figure C.4). MMP-3 immunolocalized to most umbilical cell types at 15 and 20 weeks, but by term was primarily found in the fibroblast of the Wharton’s jelly (figure C.5). MMP-9, was not identified by either western blotting or immunohistochemistry at any gestational age (not shown). During gestation, neither TIMP1 nor TIMP2 could be identified with western blotting, but immunohistochemical data suggested that TIMP2 was the predominant isoform showing a decrease in vascular smooth muscle immunoreactivity with gestation (figures C.6, C.7).
**Figure C.1**: Total MMP-1 protein expression during umbilical cord development. Panel (a) is a sample blot (n = 3 at each age group) illustrating that at 15 and 20 weeks MMP-1 is identifiable (arrow 52 kD), but shows a dramatic increase between 20 weeks and term.
Figure C.2: MMP-1 protein immunoreactivity (i.r) in the umbilical artery and Wharton’s jelly during gestation. Strong i.r. for MMP-1 was identified in both the umbilical artery (a, b) and Wharton’s jelly (e, f) between 15 weeks and term. At term, the smooth muscle cells adjacent to the lumen (a) showed strong intracellular staining for MMP-1, while further into the vascular smooth muscle media (d) MMP-1 i.r. was primarily extracellular.

Magnification a = 200 X, b = 100 X, c-f = 400X.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s jelly.
Figure C.3: MMP-2 protein activity and total protein expression during umbilical cord development. Panel (a) is a sample zymogram showing there is an increase in latent (arrow 64 kD) and active (arrow 62 kD) MMP-2 enzyme activity between 13 and 20 weeks. Panel (b) is a sample western blot showing that total MMP-2 protein expression is also increased between 13 and 20 weeks.
Figure C.4: MMP-2 protein immunoreactivity (i.r) in the umbilical artery (a, c) and Wharton’s jelly (b, d) during gestation. During gestation MMP-2 i.r was not seen in either the endothelial or vascular smooth muscle cells of the umbilical arteries (a, c). MMP-2 expression was exclusively identified in the fibroblasts of the Wharton’s jelly during gestation (b, d).

Magnification a = 400 X, b = 1000 X, c = 100 X, d = 1000 X.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s jelly.
**Figure C.5:** MMP-3 protein immunoreactivity (i.r) in the umbilical artery (a, c) and Wharton’s jelly (b, d) during gestation. In the umbilical artery MMP-3 i.r. decreased between 15 weeks (a) and term (c), but was consistently expressed in the Wharton’s jelly fibroblasts throughout gestation (b, d).

Magnification a = 400 X, b = 1000 X, c = 400 X, d = 1000 X.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s jelly.
**Figure C.6:** TIMP-1 protein immunoreactivity (i.r) in the umbilical artery (a, c) and Wharton’s jelly (b, d) during gestation. TIMP-1 i.r. was not identified in either the umbilical artery (a) or Wharton’s jelly (b) at 15 weeks, while at term TIMP-1 was exclusively expressed in the fibroblasts of the Wharton’s jelly (d).

Magnification a = 200 X, b = 1000 X, c = 100 X, d = 1000 X.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s jelly.
Figure C.7: TIMP-2 protein immunoreactivity (i.r) in the umbilical artery (a, c) and Wharton’s jelly (b, d) during gestation. TIMP-2 i.r was seen in both the umbilical artery (a) and Wharton’s jelly (b) at 15 weeks. At term, TIMP-2 i.r decreased in the umbilical artery (c), but was maintained in the Wharton’s jelly (d).

Magnification a = 200 X, b = 400 X, c = 100 X, d = 400 X.

L = Lumen, SM = Smooth Muscle, WJ = Wharton’s jelly.
Reference List


25. Dilley RJ, McGeachie JK, Prendergast FJ, A review of the proliferative behaviour,
morphology and phenotypes of vascular smooth muscle. Atherosclerosis

26. Hosoda, Y., Kawano, K., Yamasawa, F., Ishii, T., Shibata, T., and Inayama, S. Age-
Dependent Changes of Collagen and Elastin Content in Human Aorta and

27. Pawlicka E, Bankowski E, Jaworski S, Elastin of the umbilical cord arteries and its

28. Boudreau, N., Turley, E., and Rabinovitch, M. Fibronectin, Hyaluronan, and a
Hyaluronan Binding Protein Contribute to Increased Ductus Arteriosus

29. van der Rest M, Garrone R, Collagen family of proteins. FASEB J. 1991;5:2814-
2823.

30. Olsen BR, New insights into the function of collagens from genetic analysis.


Hypertension 1984;6:III44-III49

33. Burton AC. Physiology and Biophysics of the Circulation. Chicago: Year Book
Medical Publishers. 1972:

34. Ayad S, Boot-Handford RP, Humphries MJ, Kadler KE, Shuttleworth CA. The


57. Schoenberg MD, Moore RD, Studies on Connective Tissues II. Histochemical differences in the connective tissue polysaccharides of the mature and immature human umbilical cord. A.M.A. Arch. Path. 1957;64:167


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137. Sanford LP, Ormsby I, Gittenberger-de Groot AC, et al, TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development 1997;124:2659-2670.


