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A comprehensive review of the amniotic membrane and amniotic fluid

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A COMPREHENSIVE REVIEW OF THE
AMNIOTIC MEMBRANE AND AMNIOTIC FLUID

by

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B.S., Northeastern University, 2010

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DEDICATION

I would like to dedicate this work first and foremost to my spouse Jean-Marie, my best friends John, Phillip, Britton, and Abdul, and my dogs Atticus and Vinny.
ACKNOWLEDGEMENTS

I would like to thank my fiancé Jean-Marie for greatly supporting me through three years of my academic masters career. Her strength, courage and independence provided the framework for which was required to complete this thesis.

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A COMPREHENSIVE REVIEW OF THE
AMNIOTIC MEMBRANE AND AMNIOTIC FLUID

JOSEPH ANTHONY BRAZZO III

ABSTRACT

The amniotic membrane and the amniotic fluid are one of life’s most complex and delicate tissues and fluids, respectively. What was known about this tissue and fluid prior to the 20th century was extremely limited scientifically, but was significantly defined by beliefs entrenched in mysticism, folklore, and superstitions. A comprehensive literature review of the amniotic membrane tissue and amniotic fluid reveals the many unique and complex characteristics and biological properties that been heavily investigated since the turn of the 20th century and continues to surge into the 21st century. The historical perspectives, evolution, derivation, histology, structure, and composition of the amniotic membrane; and historical perspectives, volume and regulation, and cellular and non-cellular composition of the amniotic fluid are discussed here and are coalesced for an easy and comprehensible resource. Lastly, future perspectives regarding research and application of the amniotic membrane and amniotic fluid, including stem cells are discussed.
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<tr>
<td>17-OHP</td>
<td>17 α-hydroxyprogesterone</td>
</tr>
<tr>
<td>ADH</td>
<td>Antidiuretic hormone</td>
</tr>
<tr>
<td>AF</td>
<td>Amniotic fluid</td>
</tr>
<tr>
<td>AFDSC</td>
<td>Amniotic fluid-derived stem cells</td>
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<tr>
<td>AM</td>
<td>Amniotic membrane</td>
</tr>
<tr>
<td>AME</td>
<td>Amniotic membrane epithelia</td>
</tr>
<tr>
<td>AM-ESC</td>
<td>Amniotic membrane epithelial stem cells</td>
</tr>
<tr>
<td>AM-MSC</td>
<td>Amniotic membrane mesenchymal stem cells</td>
</tr>
<tr>
<td>AMSC</td>
<td>Amniotic fluid mesenchymal stem cells</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BM-MSC</td>
<td>Bone-marrow mesenchymal stem cells</td>
</tr>
<tr>
<td>CA</td>
<td>Cancer antigen</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CM</td>
<td>Chorionic membrane</td>
</tr>
<tr>
<td>DAF</td>
<td>Decay-accelerating factor</td>
</tr>
<tr>
<td>DAH</td>
<td>Dehydroepiandrosterone</td>
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<tr>
<td>DHAS</td>
<td>Dehydroepiandrosterone sulfate</td>
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<tr>
<td>E cells</td>
<td>Epitheloid cells</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
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<tr>
<td>EPO</td>
<td>Erythropoietin</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>F-Type cells</td>
<td>Fibroblast-like cells</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>HGFR</td>
<td>Hepatocyte growth factor receptor</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
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<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IGF-1</td>
<td>Insulin growth factor-1</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>iPS</td>
<td>Induced pluripotent stem cells</td>
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<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>KGFR</td>
<td>Keratinocyte growth factor receptor</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
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<tr>
<td>MIP</td>
<td>Membrane attack complex (MAC) inhibitory protein</td>
</tr>
<tr>
<td>Oct-4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>PEDF</td>
<td>Pigment epithelium-derived factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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TIMP  Tissue inhibitor of metalloproteinase
TNF   Tumor necrosis factor alpha
WJ    Wharton’s jelly
CHAPTER 1

INTRODUCTION

The amniotic sac is composed of the amniotic membrane (AM) and amniotic fluid (AF) (Larsen, 1997). It contains life’s most delicate and sensitive creation, the developing embryo and fetus (Larsen, 1997). Together the AM and AF must maintain an internal environment capable of sustaining life in the presence of a fluctuating, dynamic external environment (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005). It is with no surprise that the AM and AF are of the most complex tissues and fluids in the human body, respectively (Larsen, 1997). It is only recently within the past century have the many characteristics and biological properties of the AM and AF been discovered (Trelford & Trelford-Sauder, 1979).

Scientific investigation of the AM and AF was sparse before the 20th century (Trelford & Trelford-Sauder, 1979). Reference of the AM and AF date back to Ancient Egypt; and for centuries following, mystery, superstition and folklore defined the state of knowledge of the AM and AF (Longo & Reynolds, 2010; Crawford-Mowday, 2009; The Learning Company, 1997). The turn of the 20th century marks a major transition of the state of knowledge of the AM and AF from mystery to significant scientific inquiry (Trelford & Trelford-Sauder, 1979). A compendium of historical perspectives is pertinent to grasp the current state of knowledge and research of this tissue and fluid (Longo & Reynolds, 2010).
However, the many beliefs of the AM and AF of various cultures are both intriguing and educational (Longo & Reynolds, 2010). Historical perspectives before the 20th century and after the 20th century on the AM and AF are discussed in the beginning of their associated chapters.

A detailed review of the historical perspectives, evolution, embryology, histological structure and immunohistochemistry, and biological properties of the AM are discussed in Chapter 2. Chapter 3 includes a detailed review of the historical perspectives, volume and regulation, non-cellular composition, and non-stem cell and stem cell composition of the AF. Most importantly, in chapter 4 the future direction of research regarding this tissue and fluid are discussed.
CHAPTER 2

THE AMNIOTIC MEMBRANE

2.1 Historical Perspectives

2.1.1 Pre-20th Century

Scientifically, little was known about the AM prior to the 20th century (Trelford & Trelford-Sauder, 1979). Knowledge on the AM during this period was the product of heavy interests in and fascination for embryology, specifically the development and creation of life (Longo & Reynolds, 2010; Trelford & Trelford-Sauder, 1979). A search on pre-20th century literature on the AM reveals an acquisition of scientific knowledge non-specific for the membrane itself. More specifically, scientific knowledge on the AM was often the result of the investigation on the placenta including the chorionic membrane (CM) without specification (Longo & Reynolds, 2010; Trelford & Trelford-Sauder, 1979). For this reason historical perspectives on embryological tissues as they pertain to the AM are included in this section.

Although scientific knowledge on the AM prior to the 20th century was limited, the state of knowledge on the AM wholly was entrenched in mysticism, folklore, as was most of medicine (Longo & Reynolds, 2010; Trelford & Trelford-Sauder, 1979). This mysticism and folklore brought about superstitions and beliefs of the AM ranging from immortality to black magic (Longo & Reynolds,
Also, many religious texts throughout history make reference to the AM (Longo & Reynolds, 2010; Carr, 2004; Elsaie, 2004; Olivelle, 1998; Shakya, 1998). The mutuality of scientific knowledge, mysticism and folklore, and religious reference of the AM pre-20th century should not be overlooked (Longo & Reynolds, 2010; Trelford & Trelford-Sauder, 1979). Such mutuality provides insight into the acquisition of knowledge of the AM throughout history, and heavily emphasizes the importance and delicacy of this tissue (Longo & Reynolds, 2010; Trelford & Trelford-Sauder, 1979). Historical perspectives on the AM pre 20th century are explored chronologically.

2.1.1.1 Ancient Egypt (3100BCE–300BCE)

In Ancient Egypt the Goddess of fertility and motherhood was Isis (The Learning Company, 1997). Isis was described to possess great magical powers, including the powerful ability to create and destroy life (The Learning Company, 1997). Ancient Egyptians that placed Isis and her husband, Osiris, at the center of Egyptian deities were deemed the “cult of Isis” (The Learning Company, 1997). In early Ancient Egypt there was only a small fraction of those following the cult of Isis (The Learning Company, 1997). Popular belief among Ancient Egyptian civilization was that a baby born with the “caul” was destined for the cult of Isis (The Learning Company, 1997). Discussed below in more detail, a “caul” is the AM covering of a fetus during birth (Trelford & Trelford-Sauder, 1979).

2.1.1.2 Vedic Period (1700BCE–500BCE)
The Upanishads are the core philosophical texts of the Hindu religion (Olivelle, 1998). They are a collection of scriptures believed to teach the fundamental principles of the universe and mankind through reader introspection, inner spirituality, and philosophy (Olivelle, 1998). Writing of the Upanishads scriptures is believed to have spanned several hundred years from 800 BCE to 400 BCE (Olivelle, 1998). With over 200 Upanishads, there are, however, thirteen principle Upanishads (Olivelle, 1998). One of the thirteen principle Upanishads is the Chāndoya Upanishad (Olivelle, 1998). The Chāndoya Upanishad, “you are that”, seeks to explain the origin of the universe (Olivelle, 1998). Interestingly, in the Chāndoya Upanishad we are told that the universe developed from an egg that split in two upon hatching (Olivelle, 1998). The first half became silver, representing the Earth, and the second half became gold, representing the sky (Olivelle, 1998). We are told of the existence of two membranes of the egg, which have figurative representation, the outer membrane being the mountains, and the inner membrane, the amniotic membrane, being the clouds and mist (Olivelle, 1998). This is possible indication that the biological membranes, including the AM, were known to exist among people of Asian continent; however, we do are not informed to what extent (Olivelle, 1998).
2.1.1.3 Ancient Greece (1200BCE–300BCE)

Hippocrates of Cos is considered the father of embryology (Power & Schulkin, 2012; Longo & Reynolds, 2010). His study of embryology would establish a school of thought that would continue well into the medieval ages (Power & Schulkin, 2012; Longo & Reynolds, 2010). Hippocrates's scientific investigation of the embryology was very limited, and often being the product of philosophical logic and the study of chick embryos (Power & Schulkin, 2012). Hippocrates believed that the growing fetus would nourish itself from the suckling of “uterine paps” of the uterine wall (Power & Schulkin, 2012). No mention of the AM specifically can be found in Hippocrates’s notes on embryology (Power & Schulkin, 2012).

Many of Aristotle's discoveries in embryology would challenge Hippocrates’s embryology school of thought (Power & Schulkin, 2012; Longo & Reynolds, 2010). Aristotle’s study of embryology was heavily based on experiments and dissections of various animals, however, never humans (Power & Schulkin, 2012). Many of Aristotle’s conclusions of embryology on studied animals have been shown to be largely correct (Longo & Reynolds, 2010). Aristotle largely refuted Hippocrates’s “uterine paps” (Power & Schulkin, 2012). Using chick embryos, Aristotle showed that a chick fetus was encased in membranes and thus suckling could not be possible as claimed by Hippocrates (Power & Schulkin, 2012; Longo & Reynolds, 2010). Aristotle believed
nourishment of the fetus was through the umbilical cord directly connected to the uterus of the mother; however, he incorrectly postulated that maternal and fetal blood were one (Power & Schulkin, 2012; Longo & Reynolds, 2010). It can be said that Aristotle was the first to scientifically acknowledge fetal membranes (Power & Schulkin, 2012). However, differentiation of the AM from the CM and placenta by Aristotle is not noted (Power & Schulkin, 2012; Longo & Reynolds, 2010).

2.1.1.4 Ancient Rome (500BCE–400AD)

In Ancient Rome Claudius Galenus (Galen) of Pergamos, a Greek physician-scientist made significant contributions to the field of embryology (Longo & Reynolds, 2010). Galen was the physician to multiple Roman emperors. Not only was Galen a physician, he was also a diligent anatomist (Longo & Reynolds, 2010). Galen performed a great amount of dissections on Barbary macaques (Power & Schulkin, 2012; Longo & Reynolds, 2010). Galen’s conclusions in embryology opposed Hippocrates’s conclusions in embryology, and supported Aristotle’s (Power & Schulkin, 2012; Longo & Reynolds, 2010). Galen believed that the uterus of the mother would latch on to the CM of the developing fetus (Longo & Reynolds, 2010). There are no indications that the CM Galen refers to include the AM; however, because of the fineness of the fetal membranes the distinction of multiple membranes during this period and before was impractical (Longo & Reynolds, 2010; Trelford & Trelford-Sauder, 1979).
2.1.1.5 Medieval Period (400AD-1400AD)

The prophet Muhammad wrote the religious text of the Islamic faith, the Quran, in the early 7th century. In the noble Quran (قرآن) the word of the Lord, Allah, references indirectly the AM by indicating the creation of life developing in three veils of darkness within the womb of the mother (Elsaie, 2004). Surah 39, Ayah 6 “…He creates you in the wombs of your mothers in stages, one after another, in three veils of darkness. Such is Allah, your Lord and Cherisher. To Him belongs (all) dominion. There is no God but He: Then how are ye turned away (from your true Lord)?” (Elsaie, 2004). Scholars of the Qur’an believe the first veil is in reference to the abdominal wall of the mother, the second is the uterus, and the third is the fetal membranes, including the placenta and AM (Elsaie, 2004). Again, it is uncertain if the fetal membranes indicated in the Qur’an are distinguished during the time period in which it was written. What we do know is that Islamic scholars knew the developing fetus was encased in a membrane, which included the AM (Elsaie, 2004).

Derived from the Latin word caput galeatum literally meaning “helmeted head”, the “caul” or “born with the caul” describes the covering of the head and neck of a newborn with AM (Trelford & Trelford-Sauder, 1979). The occurrence of such a birth is exceptionally rare with a frequency of about 1 in 80,000 births (Trelford & Trelford-Sauder, 1979). Rarer is the birth of a newborn fully encased within the amniotic sac (Trelford & Trelford-Sauder, 1979). These births are
known as “en-cauls” distinct from the “caul” (Trelford & Trelford-Sauder, 1979). Mysticism, superstitions, and folklore surrounding the AM during the medieval ages can be significantly attributed to the babies born with the “caul” (Crawford-Mowday, 2009; Trelford & Trelford-Sauder, 1979). In the late medieval ages it was thought that babies born with a caul or “veil” were inherently destined for greatness, providing them with good luck and protection from intangible evil forces (Crawford-Mowday, 2009). Often, a midwife removed the caul of a newborn by pressing a piece of paper onto the caul of the head and neck (Crawford-Mowday, 2009; Trelford & Trelford-Sauder, 1979). The removed caul attached to the paper was given to the mother of the baby as an heirloom (Crawford-Mowday, 2009; Trelford & Trelford-Sauder, 1979). Sailors in the late medieval ages and early modern period highly valued cauls (Crawford-Mowday, 2009). It was believed that a caul provided good luck on sea journeys, and also gave the sailor in possession of a caul safety from death of drowning (Crawford-Mowday, 2009). Considered a sailor’s charm, many sailors paid considerable prices to possess one (Crawford-Mowday, 2009). These beliefs would continue well into the Early Modern period into the Victorian period, and even into the middle 20th century (Crawford-Mowday, 2009).

In other regions of Europe, particularly Eastern Europe it was believed that babies born with a “caul” was evidence that the baby would return from the dead (Radford, 2012). This was an indication of the birth of a vampire (Radford, 2012). Babies born with a “caul” were immediately killed or grew up and labeled by the
public with great suspicion (Radford, 2012). Also, twins born with the caul were considered demons (Radford, 2012).

2.1.1.6 Early Modern Period (1450AD–1750AD)

Leonardo da Vinci made significant contributions to the field of embryology (Gilson, 2013; Longo & Reynolds, 2010). Leonardo da Vinci studied the development of organisms in various animal species, particularly the chicken embryo (Gilson, 2013). He produced detailed drawings of the developing chicken embryos and fetuses (Gilson, 2013). These drawings are still used in embryology texts and teachings (Gilson, 2013). Most noted in Leonardo’s detailed drawings are the membranes of which the developing fetus is enclosed, including the inner AM, CM, allantois, and the umbilical and uterine vasculature (Gilson, 2013). These drawings indicate the depth of knowledge and familiarity of the fetus and embryological membranes Leonardo had acquired through his own research (Gilson, 2013). Nothing specific of the AM is noted in Leonardo’s work (Gilson, 2013; Longo & Reynolds, 2010).

The beliefs and traditions of an agrarian culture situated in the mountainous Friuli region of northern Italy held dear the power and superstitions of the caul (Ginzburg, 1983). For two centuries beginning in the 16\textsuperscript{th} century individuals of this region believed the existence of good and evil witches and warlocks in perpetual battle (Ginzburg, 1983). The good witches and warlocks known as \textit{benandanti}, “good walkers”, were armed with stalks of fennel, and bad
witches and warlocks with sorghum and wooden pallets (Ginzburg, 1983). During the night hours these two forces would intensely battle into the twilight of dawn for the fertility of the land. If the *benandanti* won, the harvest would be safe, and oppositely, if the evil spirits had won feminine would ensue and also spoil all the wine in the local villages (Ginzburg, 1983). It was believed that the souls and spirits would exit the human body at night to go forth into battle and returned prior to dawn before wakefulness (Ginzburg, 1983). The power harnessed by the *benandanti* was believed to lie in the supernatural abilities of the amnion and placenta (Ginzburg, 1983). The spirits and souls of men for which the mother preserved the caul or AM was worn about the neck and compelled to go forth into battle (Ginzburg, 1983). It was believed that these tissues of the neck safeguarded and protected the *benandanti* from the evil spirits for they did so during development of the humanly body before birth (Ginzburg, 1983).

The historical perspectives mentioned above do not represent the complete picture on all knowledge and beliefs of the amniotic membrane before the 20th century. Unfortunately, literature research regarding historical perspectives pre-20th century reveals limited and scattered information on the subject. These perspectives highlight not only the importance of extra-embryonic membranes of the placenta during these times but beliefs and knowledge of such tissues were entrenched in mysticism, folklore, and superstitions (Longo & Reynolds, 2010).
2.1.2 Post-20th Century

Scientific investigation on the AM became prominent at the beginning of the 20\textsuperscript{th} century (Trelford & Trelford-Sauder, 1979). This increase in scientific inquiry pushed aside mysticism and folklore that entrenched the state of knowledge on the AM prior (Longo & Reynolds, 2010; Trelford & Trelford-Sauder, 1979). The use of the AM as a skin graft in the 1890s marked the initial scientific investigation of the membrane at the turn of the 20\textsuperscript{th} century (Trelford & Trelford-Sauder, 1979; Davis, 1910). Scientific investigations and acquired knowledge on the AM were confined to clinical and therapeutic applications in the first half of the 20\textsuperscript{th} century (Trelford & Trelford-Sauder, 1979). EM in the 1940s allowed fine structure and cellular investigations of the amniotic membrane, revealing membrane morphology, including cellular and non-cellular entities (Trelford & Trelford-Sauder, 1979). Discoveries of the membrane's biological functions and translational properties would unfold in the latter half of the 20\textsuperscript{th} century (Dua, Gomes, King, & Maharajan, 2004). In the late 20\textsuperscript{th} century, clinical and therapeutic applications of the AM would be reinvestigated and optimized, along with new applications, since their first use in the early 1900s (Dua, Gomes, King, & Maharajan, 2004). Lastly, the discovery of amniotic membrane stem cells in the 21\textsuperscript{st} century would bring forth novel clinical therapeutic applications in various medical fields (Dobreva, Pereira, Deprest, & Zwijsen, 2010).
2.1.2.1 Initial Investigations (1890s-1940s)

In 1910 Dr. John Davis of the Johns Hopkins Hospital wrote a compendium on the literature and knowledge on skin grafting, including the many surgical approaches and various tissues he used (Davis, 1910). Dr. Davis’s amniotic membrane skin graft was the first documented clinical application of the membrane (Davis, 1910). Although the results of the membrane graft were unpromising, Dr. Davis suggested that the amniotic membrane could be of great use in skin grafts given advancement in surgical and procurement techniques (Davis, 1910). Ultimately, it would mark the transition of scientific investigation of the amniotic membrane from vague scientific research of the pre-20th century to clinical and therapeutic applications (Davis, 1910).

In 1913, using new fresh tissue preservation methods implemented by Dr. Alexis Carrel, Drs. Stern and Sabella applied AM tissue to both skin burns and ulcers (Sabella, 1913; Stern, 1913; Carrel, 1912). Drs. Stern and Sabella performed independent studies; however, they often collaborated, sharing data and strategies (Sabella, 1913; Stern, 1913). Following, scientific research on the amniotic membrane declined and would not regain inquiry for some thirty years (Dua, Gomes, King, & Maharajan, 2004; John, 2003).

The creation of an artificial vagina using AM marked the resurgence of scientific inquiry on the AM in the latter half of the first century, particularly its utilization for clinical and therapeutic applications (John, 2003). In 1934 an Italian
physician used the AM as an epidermal lining for the creation of an artificial vagina in a patient with mullerian agenesis (Dua, Gomes, King, & Maharajan, 2004; John, 2003; Brindeau, 1934; Nisolle & Donnez, 1992; Burger, 1937). The same physician would later use the AM in a more extensive construction of an artificial vagina (Dua, Gomes, King, & Maharajan, 2004; John, 2003; Brindeau, 1934; Nisolle & Donnez, 1992; Burger, 1937). Also, in 1940 AM tissue was used to prevent meningo-cerebral adhesions after lacerations to the head, including gunshot wounds, depressed fracture of the skull, and craniotomy procedures (Chao, Humphreys, & Penfield, 1940). These studies showed a reduction in meningo-cerebral adhesions and were recommended for the use of adhesion prevention in other surgeries (Chao, Humphreys, & Penfield, 1940).

The first research into clinical applications using the AM in the field of ophthalmology can be attributed to Dr. Rotth (Rotth, 1940). In 1940 Dr. Rotth replaced necrotic conjunctiva of a single eye with AM obtained from caesarian sections (Rotth, 1940). He believed the AM would be easily converted and tolerated once implanted since it was most similar to the conjunctiva membrane (Rotth, 1940). Although results of the experiment were ambiguous, Dr. Rotth encouraged further investigation (Rotth, 1940). Following, in 1946, Sorsby and Simmons replaced necrotic conjunctiva induced by caustic agents with human AM (Sorsby & Symons, 1946). The clinical study entailed the replacement of conjunctiva in patients suffering from caustic burns of the eye with success (Sorsby & Symons, 1946).
membrane grafts in caustic burns of the eye, 1946). However, further experiments using rabbits and lime as a caustic agent were unsuccessful (Sorsby, Haythorne, & Reed, 1947). Clinical and therapeutic application research of the AM in the field ophthalmology would not present itself again until 1993 (Baradaran-Rafii, Aghayan, Arjmand, & Javadi, 2007; Dua, Gomes, King, & Maharajan, 2004; John, 2003).

2.1.2.2 Identification of Amniotic Membrane Cells (1940s-1960s)

Initial description of the cellular morphology of the AME can be attributed to Bautzmann and Schroder in 1955 (Benirschke, Kaufmann, & Baergen, 2006). Bautzmann and Schroder make reference to previous studies investigating properties of AM cells five to ten years prior to their investigation, which can technically be considered initial investigations on cellular morphology of the AM; however, these studies are in German without English translations and lacking abstracts. Bautzmann and Schroder's initial investigations showed structural morphology of AME to be highly variable and composed of flat, cuboidal, and columnar cells (Benirschke, Kaufmann, & Baergen, 2006). In 1965 Thomas reported two morphologically distinct AME cell types based on ultrastructure differences reporting light and dark cell types (Benirschke, Kaufmann, & Baergen, 2006; Thomas, 1965). Following, many studies investigating the morphological structure of the AME would show differences supporting both heterogeneous and homogeneous cell populations (Thomas, 1965). Studies supporting AM cell
heterogeneity include Armstrong et al. and Wynn and French in 1968 and McCoshen et al. 1981 (Benirschke, Kaufmann, & Baergen, 2006). Studies refuting such a presence include Lister et al. 1968, Sinha et al. 1971, Hempel et al. 1972, King et al. 1980, and Sonek et al. 1991 (Benirschke, Kaufmann, & Baergen, 2006). Interestingly, it was suggested that the heterogeneity of cell morphology was the product of inappropriate fixations, and method and technique differences (Benirschke, Kaufmann, & Baergen, 2006; Hoyes, Fine structure of human amniotic epithelium following short-term preservation in vitro., 1972). Later in 2003, using enzymatic histochemistry, tracer permeability analysis, and freeze-substitution fixation, the controversial morphology of AME cells was concluded to be homogeneous (Iwasaki, Matsubara, Takizawa, Takayama, Yashiro, & Suzuki, 2003).

### 2.1.2.3 Amniotic Membrane Investigation Reemergence (1970s-1990s)

The advent of newer techniques and methods for AM handling, processing, and preservation brought about heightened research in and a reemergence for clinical and therapeutic applications of the AM (Fetterolf & Snyder, 2012; Ward, Bennett, Burgos, & Fabre, 1989; Gruss & Jirsch, 1978). AM clinical and therapeutic application potentials inquiry reemerged in the 1970s with the reinvestigation of its application in wound healing and management (Gruss & Jirsch, 1978; Colocho, Graham, Greene, Matheson, & Lynch, 1974). Following were investigative studies in chronic venous leg ulcers and healing with AM
(Ward, Bennett, Burgos, & Fabre, 1989). Also, more invasive reconstructive studies of artificial vaginas were conducted (Nisolle & Donnez, 1992; Trancer, Katz, & Veridiano, 1979; Ashworth, Morton, Dewhurst, Lilford, & Bates, 1986; Dhall, 1984).

In 1987, scientists investigated the use of AM grafts to prevent post-operative adhesions (Trelford-Sauder, Dawe, & Trelford, 1978). Application of AM grafts within the abdomen or intra-peritoneal cavity was shown to significantly reduce adhesion formation in animal models (Young, Cota, Zund, Mason, & Wheeler, 1991; Trelford-Sauder, Dawe, & Trelford, 1978). Optimum closure of large abdominal wall defects including gastroschisis and omphaloceles was largely controversial in the 1970’s (Seashore, MacNaughton, & Talbert, 1975; Gharib, Ure, & Klose, 1996). Over the course of several decades, and the utilization of various treatments and procedures to correct gastroschisis and omphaloceles, AM grafts were shown to increase closure of the wall defects (Gharib, Ure, & Klose, 1996; Rennekampff, Dohrmann, Fory, & Fandrich, 1994; Seashore, MacNaughton, & Talbert, 1975).

In 1992 a group of ophthalmological surgeons presented a series of studies to a large conference of ophthalmology specialists (Dua, Gomes, King, & Maharajan, 2004). Such studies revealed successful AM grafts in the treatment of several ocular disorders, and novel procurement procedures (Dua, Gomes, King, & Maharajan, 2004; John, 2003). Following, in 1995 Kim and Tseng,
influenced by the aforementioned study above, would set the stage and successfully reintroduce AM application investigations in ophthalmology (Baradaran-Rafii, Aghayan, Arjmand, & Javadi, 2007; Dua, Gomes, King, & Maharajan, 2004; John, 2003). The treatment of various ophthalmological diseases using AM had been optimized and utilized since the study by Kim & Tseng in 1995 (Baradaran-Rafii, Aghayan, Arjmand, & Javadi, 2007; Dua, Gomes, King, & Maharajan, 2004; John, 2003). These are discussed in detail in the translational development section below. Most importantly, the many biological properties of the AM that we currently know have come from the intensive study and research on AM clinical and therapeutic applications in ophthalmology during this reemergence period (Dua, Gomes, King, & Maharajan, 2004; John, 2003).

2.1.2.4 Identification of Amniotic Membrane Stem Cells (2000s-present)

Stem cells of the AM can be placed into two categories: amniotic membrane epithelial stem cells (AM-ESC) and amniotic membrane mesenchymal stem cells (AM-MSC) (Dobreva, Pereira, Deprest, & Zwijsen, 2010; Miki, 2011). Prior to 2004, the isolation, and properties and characterization of stem cells within the membrane itself were largely unknown (Dobreva, Pereira, Deprest, & Zwijsen, 2010; Miki, 2011). The first indication that stem cells were present in the AM was in 2004 by Tamagawa et al., in which the authors claimed that cell lines from human AM contributed to all three germ layer formation in
xenogeneic mouse embryos mixed with embryonic stem cells (Miki, 2011; Dobreva, Pereira, Deprest, & Zwijsen, 2010). However, this study was unable to specify whether such a contribution and differentiation was due to that of AM-ESC or AM-MSC (Miki, 2011).

In 2005 AME cells were shown to differentiate into cells of all three germ layers further supporting a presence of stem cells specifically in the amniotic epithelial membrane, along with a subpopulation of amniotic epithelial cells expressing stem cell markers, supporting the characterization of AM-ESC (Dobreva, Pereira, Deprest, & Zwijsen, 2010; Miki, 2011). The first study initially characterizing AM-MSC is unclear (Miki, 2011; Dobreva, Pereira, Deprest, & Zwijsen, 2010). A study in 2007 done by Alviano et al. suggested that there was a presence of specific AM-MSC within the stromal layers of the AM by showing an immunophenotypical profile of AM mesenchymal cells most consistent with bone-marrow mesenchymal stem cells (BM-MSC) (Dobreva, Pereira, Deprest, & Zwijsen, 2010). Further characterization and profiling of stem cells of AM-EMS and AM-MSC would occur following their initial characterization within their specific tissue layer and location (Dobreva, Pereira, Deprest, & Zwijsen, 2010). Translational developments, particularly therapeutic and clinical applications would be equally pursued, which would utilize the properties of AM-ESC and AM-MSC (Dobreva, Pereira, Deprest, & Zwijsen, 2010). These are discussed in the translation development section below.
2.2 Evolution

The beginning of biological life took form in the vast oceans of a young earth (Miller, 1953). Over the span of hundreds of millions of years the evolution of complex, self-functioning cells derived from simple, self-replicating molecules paved the way for highly structured, sophisticated organisms (Miller, 1953). These organisms flourished in the deep blue ocean, constantly bathed and nourished by a maternal fluid (Miller, 1953). In time the evolution and development of land-dwelling animals emerged from aquatic life (Blackburn & Flemming, 2009; Leal & Ramirez-Pinilla, 2010; Pillai, 2012). The harsh environment of dry land called for adaptations suitable for life away from the nourishment of a ubiquitous fluid environment (Blackburn & Flemming, 2009) (Pillai, 2012). In particular, the sustainability and safety of the developing offspring was vitally important in the transition from aquatic to terrestrial life (Blackburn & Flemming, 2009). It was the evolution of a protective egg, the amniotic egg, roughly 340 million years ago that allowed this transition (Pillai, 2012).

Amniotes, as defined by evolutionary biologists, are land-dwelling tetrapods that produce offspring eggs, or amniotic eggs in which the embryo develops (Pillai, 2012; Leal & Ramirez-Pinilla, 2010). Amniotes envelop several phylogenetic classes including Reptilia, Aves, Mammalia, and their fossil ancestors, dinosaurs (Pillai, 2012; Leal & Ramirez-Pinilla, 2010; Blackburn & Flemming,
Within the amniote classification exist egg-laying, oviparous tetrapods, including reptiles, birds, early mammals, and fossil ancestors; and egg-bearing, viviparous tetrapods that includes late mammals (Pillai, 2012; Leal & Ramirez-Pinilla, 2010; Blackburn & Flemming, 2009). Some exceptions do exist regarding this classification but are beyond the scope of this paper (Pillai, 2012).

Characteristic of the amniotic egg is four extra-embryonic membranes (Pillai, 2012; Blackburn & Flemming, 2009). These extra-embryonic membranes include the CM, allantois, yolk sac, and the AM (Pillai, 2012; Blackburn & Flemming, 2009). The CM is the thickest, outer-most extra-embryonic membrane responsible for gas exchange (Pillai, 2012; Blackburn & Flemming, 2009; Larsen, 1997). The allantois grows out of the hindgut and stores and disposes of nitrogenous waste (Pillai, 2012; Blackburn & Flemming, 2009; Larsen, 1997). The yolk sac develops from the mid-gut, and provides the necessary nutrients for the developing organism (Pillai, 2012; Blackburn & Flemming, 2009; Larsen, 1997). Of the extra-embryonic membranes, it is the AM that is most intimate with the developing embryo and fetus, encasing the embryo and fetus in AF (Blackburn & Flemming, 2009; Larsen, 1997). Over the course of amniote evolution the functions and structure of these extra-embryonic membranes would differ with the progress of new amniote species (Pillai, 2012; Leal & Ramirez-Pinilla, 2010; Blackburn & Flemming, 2009). More fit and efficient reproductive strategies would drive such evolution of the amniotic egg (Pillai, 2012; Leal & Ramirez-Pinilla, 2010; Blackburn & Flemming, 2009). Unfortunately, the
understanding and knowledge of the diversity and evolution of amniote’s extra-embryonic membranes is extremely limited with much of our understanding coming from research of extra-embryonic membranes done on reptiles (Leal & Ramirez-Pinilla, 2010; Blackburn & Flemming, 2009).

The first amniotic eggs were of oviparous tetrapods, and contained a hardened, calcareous shell that prevented egg desiccation, and provided protection for the delicate extra-embryonic membranes and developing embryo (Leal & Ramirez-Pinilla, 2010; Blackburn & Flemming, 2009). Primitive amniotic eggs relied exclusively on the yolk sac for nutrition (Pillai, 2012; Leal & Ramirez-Pinilla, 2010; Blackburn & Flemming, 2009). In more evolved amniotes, the yolk sac serves a lesser purpose, and is less developed in placentotrophic amniotes, in which the placenta provides nutrients (Pillai, 2012; Blackburn & Flemming, 2009). An umbilical cord serves as a conduit between the fetus and placenta (Pillai, 2012; Larsen, 1997). The umbilical cord is a specialized tissue composed of the AM, CM, yolk sac, and allantois (Pillai, 2012; Larsen, 1997). This specialized tissue is discussed in more detail in the embryology section below. The allantois is the last extra-embryonic membrane to develop of the four fetal extra-embryonic membranes (Pillai, 2012; Larsen, 1997). The allantois develops complementary to the amnion (Pillai, 2012; Larsen, 1997). In oviparous amniotes the allantois grows into the chorionic cavity, serving as a site of nitrogenous waste disposal (Pillai, 2012; Blackburn & Flemming, 2009). In more evolved amniotes, the allantois fuses with CM to produce a single membrane, in
which the allantois plays a major role in gas exchange (Pillai, 2012; Blackburn & Flemming, 2009; Larsen, 1997). The transition of amniotes from oviparous to viviparous, or the fusion of membranes to create more complex tissues is unclear but is believed to be the result of more specialization of the extra-embryonic membranes (Pillai, 2012; Leal & Ramirez-Pinilla, 2010; Blackburn & Flemming, 2009).

It is important to note the enormous diversity of amniotes compared to non-amniotes, or an-amniotes (Pillai, 2012). Such diversity is seen in the size, structure, and function of amniotes (Pillai, 2012). The specialized functions of the extra-embryonic membranes found in the amniotic egg have served more than physiological and protective purposes and have brought about great diversity of species over hundreds of millions of years (Pillai, 2012; Blackburn & Flemming, 2009). There is a large gap in the knowledge in the biology of this evolutionary diversity (Pillai, 2012). Function of the AM of the amniotic egg has remained constant throughout its evolution hundreds of millions of years ago (Pillai, 2012; Leal & Ramirez-Pinilla, 2010; Blackburn & Flemming, 2009). It serves to protect the developing embryo and fetus from physical disturbances, and maintains a constant internal fluid, and nourishing environment for the developing embryo and fetus (Pillai, 2012; Blackburn & Flemming, 2009).
2.3 Embryology

The AM, or amnion derives from primary ectoderm, also known as epiblast or ectoblast cells, of the bilaminar germ disc present in the second week of gestation in humans (Larsen, 1997). The aforementioned bilaminar germ disc is part of the inner cell mass that composes part of the developing blastocyst (Larsen, 1997). Upon implantation of the blastocyst into the wall of the mother’s uterus, the inner cell mass of the blastocyst differentiates into two cellular layers: a dorsal layer of columnar cells, the primary ectoderm, mentioned above; and a ventral layer of cuboidal cells, the primary endoderm, also known as the hypoblast or endoblast cell layer (Larsen, 1997). These two primary layers of cells are called the embryoblast, or embryonic disc, and makeup all tissues of the developing embryo (Larsen, 1997).

On day 8 post-fertilization, epiblast cells begin excreting a fluid, producing a cavity, the pro-amniotic cavity, within, or a hollowing out of the epiblast layer (Larsen, 1997). This cavity is the beginning of the amniotic cavity (Larsen, 1997). As the cavity begins to grow via epiblast proliferation, cavitation of proliferating cells and apical excretion, a layer of ventral epiblast cells differentiates into a thin membrane separating the now amniotic cavity from the adjacent, more ventral cytотrophoblast (Larsen, 1997). These specialized epiblast cells, amnioblasts, are derived from the proximal-anterior epiblast cells of the newly formed bilaminar germ disc, prior to the pre-streak and primitive streak stages (Larsen,
Epiblast cells adjacent and ventral to the hypoblast but not bordering the cytotrophoblast, and continuous with the amnioblasts, are to become the intraembryonic ectoderm and mesoderm of the three-layered germ disc in week five of gestation (Larsen, 1997). These differentiated, specialized cells now known as amnioblasts makeup the inner layer of the AM and create AF during early human embryo development (Larsen, 1997). Production and composition of AF is a function of amnioblasts and are discussed in the AF volume and regulation, and composition sections of this thesis.

On day 9 post-fertilization, as the epiblast proliferates and cells are displaced by a growing amniotic cavity, the cells of the primary endoderm (hypoblast) simultaneously proliferate and line the blastocyst cavity, adjacent to the developing trophoblast, creating the exocoelomic membrane, or Heuser’s membrane (Larsen, 1997). These cells differentiate into flattened, squamous cells by day 12 post-fertilization, completely lining the once blastocoele, and define the primary yolk sac or exocoelomic cavity (Larsen, 1997). These cells are now considered extraembryonic endoderm, and are continuous with the differentiating hypoblast at the embryonic pole (Larsen, 1997).

Appearing between days 12 and 13 post-fertilization is a thick, loose reticular acellular layer called the extraembryonic reticulum (*magma reticulare*) that forms between the extraembryonic endoderm and adjacent cytotrophoblast (Larsen, 1997). Within the extraembryonic reticulum are growing lacunae (Larsen,
These lacunae grow, accumulate and coalesce, forming the extraembryonic coelom, also known as the chorionic cavity (Larsen, 1997). As the chorionic cavity grows in size, the primitive yolk sac is pinched ventrally and becomes the exocoelomic cyst (Larsen, 1997). The new dorsal part of the primary yolk sac becomes the definitive yolk sac (Larsen, 1997). It is believed that the extraembryonic mesodermal tissue lining the extraembryonic coelom is derived from proliferating and migrating posterior and posterolateral epiblast cells of the embryoblast (Larsen, 1997). The inner lining of the extraembryonic coelom is the extraembryonic mesoderm known as extraembryonic splanchnopleuric mesoderm, which is adjacent to the endoderm of the newly established definitive, secondary yolk sac and the AM (Larsen, 1997). It is believed that the endodermal cells lining the newly established definitive, secondary yolk sac is derived from newly proliferating and differentiated cells of the hypoblast, different from the cells of Heuser’s membrane although the same site of origin (Larsen, 1997). A small diverticulum forms at the posterior part of the secondary yolk sac and extends into the body stalk; this is the beginning of the developing allantois (Larsen, 1997).

The outer layer of the extraembryonic mesoderm, also called the extraembryonic somatopleuric mesoderm lines the inner layer of the cytotrophoblast and is considered the chorionic membrane (Larsen, 1997). Suspended in the newly formed extraembryonic coelom are the amniotic cavity, embryoblast, and definitive yolk sac, and bordered by the extraembryonic
splanchnopleuric mesoderm (Larsen, 1997). It is the connecting/body stalk of extraembryonic mesoderm that connects these developing structures with the developing extraembryonic trophoblast (Larsen, 1997). As the extraembryonic coelom grows larger in size, lacunae part the connecting stalk and thus separate the developing embryo and amnion from the extraembryonic trophoblast (Larsen, 1997).

Throughout gestation the amniotic and chorionic cavities grow in size (Larsen, 1997). However, as pregnancy progresses the relative size of the amniotic cavity will grow larger in ratio to that of the chorionic cavity (Larsen, 1997). As the suspended amniotic sac and embryo grow within the chorionic cavity, the volume of the extraembryonic coelom decreases in size, and the AM with the inner membrane of the chorion (extraembryonic mesoderm/reticulum) eventually fuses (Larsen, 1997). The AM is believed to completely fuse with the inner CM by the 12th week of gestation (Larsen, 1997). The fusion of the AM and CM represent an intimate association of membranes than the actual histological fusion of two membrane entities, as indicated by the easy separation of the two membranes (Larsen, 1997).

The umbilical cord, an organ found in placentotrophic, viviparous mammals including humans, mentioned above in the evolution of the AM, is a specialization of the secondary yolk sac, allantois, and connecting/body stalk of the extraembryonic mesoderm (Pillai, 2012; Larsen, 1997; Blackburn &
Flemming, 2009). The allantois is an out-pouching of the hindgut composed of extraembryonic somatoplueric mesoderm that expands into the chorionic, extraembryonic mesoderm of the connecting/body stalk (Pillai, 2012; Larsen, 1997). The extraembryonic somatoplueric mesoderm of the developing allantois fuses with the extraembryonic splanchnopleuric mesoderm of the primitive chorionic connecting/body stalk, creating the chorio-allantoic membrane (Pillai, 2012; Larsen, 1997). Upon fusion of the allantois membrane and primitive CM, the extraembryonic mesoderm of the body stalk becomes heavily vascularized (Pillai, 2012; Larsen, 1997). Developing from this heavy vascularization are the vascular conduits of the primitive umbilical cord (Pillai, 2012; Larsen, 1997).

Lining the developing umbilical cord is the AM that expands and fuses with the chorion proper in the 12th week of gestation (Larsen, 1997). However, prior to complete fusion with the chorionic proper, the AM and cavity expand and fold within the chorionic cavity (Larsen, 1997). The expanding AM first fuses with the chorionic extraembryonic mesoderm of the primitive umbilical cord closest to the developing embryo (Larsen, 1997). As gestation progresses, the AM grows outward, fusing dorsally with the umbilical cord until it completely closes and fuses with the chorionic proper (Larsen, 1997).

2.4 Structure: Histology and Immunohistochemistry

Upon microscopic examination of the AM, five distinct principal layers are revealed (John, 2003; Bourne, 1962). The first layer is a simple epithelial layer
that is in direct contact with the AF, also known as the AME (John, 2003; Bourne, 1962). The second layer is the BM, which lies between the AME layer and compact layer (John, 2003; Bourne, 1962). BM is a continuous connective tissue stroma that is divided into three layers, third through fifth, which is intimate with connective tissue of the chorionic membrane (John, 2003; Bourne, 1962). The third through fifth layers include the compact layer, fibroblast layer, and spongy layer, respectively (John, 2003; Bourne, 1962).

The extent and thickness of these layers vary depending on the location of the AM (van Herendael, Oberti, & Brosens, 1978). Three distinct sections of the AM exist: the amniotic reflectum, umbilical amnion, and placental amnion (van Herendael, Oberti, & Brosens, 1978). It is the five layers of the amniotic reflectum that will be discussed in detail for it is this structural section of the AM that is most referenced and studied (van Herendael, Oberti, & Brosens, 1978). Slight variations of these layers do exist among the placental amnion and umbilicus amnion and are discussed accordingly (van Herendael, Oberti, & Brosens, 1978). Also, the immunohistochemistry of each layer is described to provide a more detailed analysis of the structure of such layers.

Temporally, the cellular morphology of the AM changes, but is relatively constant from about the fourth month of gestation until birth (Benirschke, Kaufmann, & Baergen, 2006; Pollard, Aye, & Symonds, 1976). Much of the literature on the structure and morphology of the amniotic membrane is limited to
the amniotic reflectum of term births (van Herendael, Oberti, & Brosens, 1978). The structure of the AM throughout gestation is briefly discussed with reference to the AM lining the umbilical cord, umbilical amnion, and AM lining the chorionic leave of the placenta, placental amnion (van Herendael, Oberti, & Brosens, 1978).

2.4.1 Amnion Reflectum

2.4.1.1 Epithelial Layer

Light microscopy of delivered term AM reflectum epithelial reveals consistent, homologous staining cells with moderately staining, centrally located nuclei (Bourne, 1962). Epithelial of the amniotic reflectum ranges in morphological shape from cuboidal to columnar (Benirschke, Kaufmann, & Baergen, 2006). Columnar cells appear where the AM reflectum inserts into the placental margin (Benirschke, Kaufmann, & Baergen, 2006). It is important to note that AM derived from caesarian sections show an AME layer of cells that are uniformly cuboidal in shape compared to the greater diversity in morphological shape found in AM of normal, labor deliveries (Thomas, 1965). The apical surface, intimate with the AF, is convex in shape (Bourne, 1962). A more detailed description is given by EM. Also, thickness of the AME ranges from 18-56μm depending on location of the AM with thickness being greatest at the umbilicus closest to the fetus (Benirschke, Kaufmann, & Baergen, 2006).
Ultrastructure examination by Thomas revealed two different cell types of AME: light and dark cell types (Thomas, 1965). It was therefore suggested that light and dark cell types had different roles as AME cells. The first is the “Golgi Type” dark cell type (Thomas, 1965). Ultrastructurally, the “Golgi type” cell type has mitochondrial distributed throughout the cell preferably around the nucleus and towards the basal side (Thomas, 1965). There are significant amounts of lipid granules that are ubiquitous throughout the cell (Thomas, 1965). The strongest feature of this cell type is an extensive and widely distributed Golgi apparatus, and apparent rough and smooth endoplasmic reticula, suggesting a large secretory role of this cell type (Thomas, 1965). Also, complex foot processes are found at the basal surface of this cell type (Thomas, 1965). The second cell type is the “Fibrillar Type” light cell type61. According to Thomas, this cell type is denser in appearance than the “Golgi Type” cell with dense fibrils arranged in random orientation within the cell (Thomas, 1965). There is a smaller concentration and dispersion of mitochondria, Golgi apparatus, and endoplasmic reticulum suggesting a less important secretory role than that of the “Golgi Type” cell (Thomas, 1965). Cell types described by Thomas revealed a heterogeneous mixture of cell types of AME (Thomas, 1965). Following Thomas’s results, studies would support or refute such findings (Benirschke, Kaufmann, & Baergen, 2006; Hoyes, Fine structure of human amniotic epithelium following short-term preservation in vitro., 1972). More specifically, Lister et al. described the ultrastructure of a single, homogeneous AME layer scarce of apical microvilli,
complex intercellular microvilli with desmosomes, large nuclei, abundant glycogen, and infrequent mitochondria, endoplasmic reticulum, and Golgi lamellae (Benirschke, Kaufmann, & Baergen, 2006). These similarities represent universal morphological structure features of these cells, which are discussed below.

EM reveals an ultrastructure of the AME to have numerous microvilli structures on the apical end of the epithelial cells (Benirschke, Kaufmann, & Baergen, 2006; Sinha, 1971). These microvilli are of a short, blunt, and simple branched type (Sinha, 1971). They have a surface with a densely packed glycocalyx (Benirschke, Kaufmann, & Baergen, 2006). Morphology of the microvilli is identical of both cell types mentioned above: “Golgi Type” and “Fibrillar Type cell types (Thomas, 1965). Also, microvilli are found to continue down the lateral side of AME cells and form intercellular channels (van Herendael, Oberti, & Brosens, 1978).

AME nuclei are centrally located, and are irregular in shape with fenestrations (Bourne, 1962). Vacuoles are apparent within the cytoplasm (Bourne, 1962). These vacuoles are most prevalent at the lateral portions of AME, and with limited vacuoles present in the apical, microvilli portion of the cell (Bourne, 1962). Also, there is a large presence of vacuoles closely associated with the nucleus of the cell (van Herendael, Oberti, & Brosens, 1978). These vacuoles have been shown to contain lipid substances (van Herendael, Oberti, &
Some vacuoles of adjacent cells appear to be connected via fine channels (Bourne, 1962). Channels and canals are believed to be part of a large, extensive communication system between cells of the AME (Bourne, 1962). Numerous desmosomes can be found between adjacent AME cells in which the vast arrays of filaments within the cytoplasm are connected (Bourne, 1962).

AME cells have been shown to express CA-1, CA-2, and CA-125, peptidase dipeptidylpeptidase IV, HLA class I molecules, EGF receptors, TGF-β1, TGF-β2, TGF-β3, and their type I and II receptors, CD44 (HA receptor), desmin, oxytocin receptors, erythropoietin and erythropoietin receptors; but do not express HLA class II molecules (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012; Dua, Gomes, King, & Maharajan, 2004). These cells can be induced to express ICAM-1, TNF-α, and IL-1β (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012; Dua, Gomes, King, & Maharajan, 2004).

Immunohistochemistry of the cytoskeleton of AME cells reveals the expression of various cytoskeleton proteins (Wolf, Schmidt, & Drenckhahn, 1991). Distributed preferentially toward apical and lateral surfaces of AME cells include actin, α-actinin, spectrin, and ezrin (Wolf, Schmidt, & Drenckhahn, 1991). However, distributed universally throughout AME cells’ cytoplasm is the protein vimentin (Wolf, Schmidt, & Drenckhahn, 1991). The architecture of the cytoplasm’s cytoskeleton and various lateral cellular junctions are believed to
play an important role in diffusion between maternal circulation and AF (Wolf, Schmidt, & Drenckhahn, 1991).

2.4.1.2 Basement Membrane

Light microscopy (LM) of the BM reveals very little due to the inherent nature of its small size. For this reason EM and immunohistochemistry are required to assess the BM in more detail.

AME cells are attached to a thick BM (Benirschke, Kaufmann, & Baergen, 2006). This BM is considered to be the thickest of all BM found in human tissues (Benirschke, Kaufmann, & Baergen, 2006). A typical BM is composed of a basal lamina and lamina reticularis, which can be discerned using EM (Aplin, Campbell, & Allen, 1985). More specifically, the basal lamina is composed of two distinct layers, the lamina lucida and the lamina densa (Aplin, Campbell, & Allen, 1985). Ultrastructure examination of the AM BM reveals three different layers of varying size fibrils (Bourne, 1962). The first layer closest to the AME layer is composed of a network of delicate, parallel fibers of type IV collagen (Aplin, Campbell, & Allen, 1985; Bourne, 1962). This layer most likely represents the delicate fiber type found in the lamina lucida of most basal laminae (Aplin, Campbell, & Allen, 1985). This fibrous network runs from the lamina densa to the basal cell surface of the AME (Aplin, Campbell, & Allen, 1985). This layer is most consistent with the lamina lucida of the BM (Aplin, Campbell, & Allen, 1985; Thomas, 1965). Below, the second layer is composed of a looser network of larger fibers of the
reticular and collagen types (Aplin, Campbell, & Allen, 1985; Thomas, 1965). The second layer is consistent with the lamina densa of the BM (Aplin, Campbell, & Allen, 1985; Thomas, 1965). The third layer of the BM, is composed of large collagen fibers arranged in random orientation (Thomas, 1965; Bourne, 1962). It is not clear if this third layer is a continuation of the second BM layer, lamina lucida, or is in fact the lamina reticularis of the BM (Thomas, 1965; Bourne, 1962). Collagen type I, and fibronectin are found in all layers of the BM (Aplin, Campbell, & Allen, 1985).

Large amounts of proteoglycans with heparin sulfate, collagen types I, III, IV, V, and VII, laminin, and fibronectin can be found in the BM (Dua, Gomes, King, & Maharajan, 2004). Hemidesmosomes can be found associated with the first layer of the BM, the lamina lucida, and attached to the basal surface of AME cells (Dua, Gomes, King, & Maharajan, 2004). Integrins that make up such hemidesmosomes include α6β4, and B-1 integrins (Dua, Gomes, King, & Maharajan, 2004).

2.4.1.3 Compact Layer

The first layer of the AM mesenchymal stroma is the compact layer (Dua, Gomes, King, & Maharajan, 2004). This layer is avascular and devoid of cells (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012; Dua, Gomes, King, & Maharajan, 2004; Bourne, 1962). It is believed that the compact layer is the strongest of all 5 layers, and is responsible for maintaining the tensile and
mechanical properties of the AM (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012). It is the arrangement of collagen fibers and size that is believed to give such tensile properties to the AM not elastin, which is scare in the AM (Baradaran-Rafii, Aghayan, Arjmand, & Javadi, 2007).

EM of the compact layer of the AM reveals a dense packing of collagen fibers (Aplin, Campbell, & Allen, 1985). A transition zone can be seen within the compact layer (Aplin, Campbell, & Allen, 1985). Above the transition zone, closets to the BM, is a tightly packed network of fibers; however, they are small in diameter (Aplin, Campbell, & Allen, 1985). Below the transition zone is a thicker class of fibers but are less tightly packed than fibers above the transition zone (Aplin, Campbell, & Allen, 1985). These fibers found in the compact layer are prominently composed of collagen type I and III fibers (Bourne, 1962). Also, small amounts of parallel bundles of collagen types II, IV and V can be found in the compact layer (Aplin, Campbell, & Allen, 1985). Thickness of the compact layer ranges from 5-20μm (Bourne, 1962). It has been suggested that this layer, rich in collagen type III fibers (reticular fibers) is a large lamina reticularis, a continuation of the thick BM of the AM (Ockleford, et al., 1993). It is possible that this layer represents, or is a continuation of the third layer of the BM mentioned above (Thomas, 1965; Bourne, 1962; Ockleford, et al., 1993). Synthesis of collagen types I, III, and IV; and laminin and fibronectin by AME cells makes up the fibers that create their BM (Aplin, Campbell, & Allen, 1985). They create the fibers that make up the BM of the AM and compact layer suggesting that the
compact layer is indeed a unique large lamina reticularis of the BM of the AM (Ockleford, et al., 1993).

Several fibronectin isoforms can be found in the compact layer of the AM, which are secreted by AME cells (Benirschke, Kaufmann, & Baergen, 2006). These molecules compose part of the meshwork in which the dense collagen type III fibers of the compact layer are found (Campbell, Allen, Moser, & Aplin, 1989).

### 2.4.1.4 Fibroblast Layer

Below the compact layer is the fibroblast layer (John, 2003; Bourne, 1962). This layer is the thickest of the 5 layers of the AM (Benirschke, Kaufmann, & Baergen, 2006). This layer is composed of a collagen matrix, reticular fibers and branched fibroblast-like mesenchymal cells also referred to as AM mesenchymal cells (Baradaran-Rafii, Aghayan, Arjmand, & Javadi, 2007). This layer is avascular (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012). Cells of the AM mesenchymal stroma derive nutrients through diffusion from AF on one side and the vascular CM on the opposite side (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012; Benirschke, Kaufmann, & Baergen, 2006).

It is this large array of reticular fibers found in the fibroblast layer that gives the AM great elasticity (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012). No tight junctions can be found between branched fibroblast cells within
this layer (Bou-Resli, Al-Zaid, & Ibrahim, 1981). Also, macrophages or Hofbauer cells can be found scarcely throughout this layer and the spongy layer below (Bourne, 1962). Ultrastructure examination of fibroblast cells reveals a single nucleus with varying diameter and eccentrically located, a foamy or vacuolated cytoplasm (Bourne, 1962). Pseudopodia can be seen occasionally suggesting active phagocytic events (Bourne, 1962). The cell itself is oval to round in shape (Bourne, 1962). They have been shown to contain large quantities of meconium in their vacuoles (Bourne, 1962).

Characterization of AM mesenchymal stroma cells is defined by fibroblast-like cell morphology are positive for cell surface markers CD13, CD29, CD44, CD54, CD73, CD105, and CD166 (Toda, Okabe, Yoshida, & Nikaido, 2007).

2.4.1.5 Spongy Layer

Adjacent to the fibroblast layer of the AM is the spongy layer (Benirschke, Kaufmann, & Baergen, 2006). This layer is considered to be the transition or intermediate between the AM and CM (Benirschke, Kaufmann, & Baergen, 2006). The spongy layer is largely composed of hydrated proteoglycans and glycoproteins, and non-fibrillar type III collagen (reticular fibers) (Benirschke, Kaufmann, & Baergen, 2006; Bourne, 1962). Collagen of the reticular type is present in wavy bundles (Bourne, 1962). This layer varies in thickness due to absorptive properties due to the heavy presence of proteoglycans and glycoproteins, and allows movable properties that do restrict AM attachment to
the CM allowing sliding against the CM (Benirschke, Kaufmann, & Baergen, 2006). Below the spongy layer is the mesenchymal stroma, or connective tissue of the CM (Benirschke, Kaufmann, & Baergen, 2006). Here we find a reticular layer that contains CM mesenchymal cells that have been shown to resemble hematopoietic progenitor cells (Benirschke, Kaufmann, & Baergen, 2006). This layer is avascular in the first trimester but becomes vascularized in the beginning of the second trimester (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012; Benirschke, Kaufmann, & Baergen, 2006). Occasional fibroblast and macrophages, described above are found within the spongy layer (Bourne, 1962). Collagen types I and IV can also be found in this layer (Niknejad, Peirovi, Jorjani, Ahmadiani, Ghanavi, & Seifalian, 2008).

The ultrastructure of the spongy layer of the AM reveals a unique geometry of the reticular fibers as being branched with triangular shaped nodes at the junctions (Bourne, 1962). These fibers are also loosely arranged separated by a system of clefts believed to be the remainder of the once large exocoel (Benirschke, Kaufmann, & Baergen, 2006).

2.4.2 Umbilical Amnion

The structural morphology of the AME lining the umbilical cord varies by location and with gestational age (Hoyes, Ultrastructure of the epithelium of the human umbilical cord., 1969). Between 8 and 10 weeks of gestation the AME of the umbilical cord is a single layer of squamous cell (Hoyes, Ultrastructure of the
epithelium of the human umbilical cord., 1969). At about 10 to 15 weeks of
gestation the amniotic epithelial of the umbilicus is bilaminar (Hoyes,
Ultrastructure of the epithelium of the human umbilical cord., 1969). The later
part of the 5th month, including the sixth and 7th months of pregnancy, three or
more layers can be found, increasing in number with gestational age (Hoyes,
Ultrastructure of the epithelium of the human umbilical cord., 1969). Some parts
of the AME of the umbilicus are four or five layers thick (Hoyes, Ultrastructure of
the epithelium of the human umbilical cord., 1969). Lower layers of the stratified
epithelium stain darker than top layers (Hoyes, Ultrastructure of the epithelium of
the human umbilical cord., 1969).

The progression and differentiation of the AM that lines the umbilical cord
is similar to that of the fetal epidermis; however, there is no evidence of definitive
keratinization in the majority of squamous epithelial of the umbilicus AM (Hoyes,
Ultrastructure of the epithelium of the human umbilical cord., 1969). However,
keratinization has been found of the squamous epithelia of the umbilical cord AM
closest to the fetus (Hoyes, Ultrastructure of the epithelium of the human

Ultrastructurally, organelles and glycogen are apparent in the apical
cytoplasm of the superficial cells from week 11 to 26 of gestation (Hoyes,
Ultrastructure of the epithelium of the human umbilical cord., 1969). These
findings indicate a secretory role of AME of the umbilical cord within these weeks
These cells contain relatively few microvilli, and there are few villous folds of the AME (Sinha, 1971). However, cilia are present on the apical cell surface of AME cells (Hoyes, Ultrastructure of the epithelium of the human umbilical cord., 1969).

2.4.3 Placental Amnion

AME cells lining the placental CM are cylindrical in shape (van Herendael, Oberti, & Brosens, 1978). Microvilli of the placental AM are shown to be interwoven among adjacent apical cells (van Herendael, Oberti, & Brosens, 1978). The nuclei of these AME cells are located closer to the apical portion of the cells compared to AME cells of the amniotic reflectum, which are centrally located (van Herendael, Oberti, & Brosens, 1978). BM foot processes are prominent of the placental AME (van Herendael, Oberti, & Brosens, 1978).

2.5 Functions and Properties

The AM is more than a simple, static membrane (Benirschke, Kaufmann, & Baergen, 2006). It serves not only as a physical barrier, but protects the delicate fetus from physical trauma, and is metabolically and physiologically active both preventing desiccation of the fetus, and maintaining a dynamic, living solution in which the fetus develops (Benirschke, Kaufmann, & Baergen, 2006). The many properties of the AM unraveled from decades of research have given new promises in various medical fields through translational developments, mainly clinical and therapeutic applications (Mamede, Carvalho, Abrantes,
The discovery of many of these properties can be most attributed to research done in ophthalmology wishing to utilize the AM for optical diseases and disorders done over the past two decades (Dua, Gomes, King, & Maharajan, 2004). These properties are discussed below.

2.5.1 Physical Properties

The AM is a membrane of great strength (Benirschke, Kaufmann, & Baergen, 2006). Given its relative thickness the AM is able to withstand great trauma, including shear force and stretching (Benirschke, Kaufmann, & Baergen, 2006). These properties are inherent within the histological make-up of the membrane (Benirschke, Kaufmann, & Baergen, 2006; Lavery, Miller, & Johns, 1980; Wyatt-Ashmead & Ashmead, 2004). The average tensile strength of the AM is 2-5 g/cm with a range of 50-500 g/cm (Lavery, Miller, & Johns, 1980). The tensile strength of the AM is greater than that of the CM (Wyatt-Ashmead & Ashmead, 2004). Also, the AM tensile strength is greatest during early gestation and decreases towards term; however, the tensile strength of the AM at term is greater than that required for successful parturition (Wyatt-Ashmead & Ashmead, 2004).

2.5.2 Immunological

It was suggested by Talmi et al. that the AM contained no inherent anti-microbial properties but such properties exhibited by the membrane were due to
the membrane’s ability to adhere firmly and sound to substances and thus prevent penetration by bacteria and infection (Dua, Gomes, King, & Maharajan, 2004). Research investigating the antiviral properties of the AM is limited. It is known that the AM contains the protein cystatin E, a proteinase inhibitor analogous to cysteine proteinase inhibitor with known antiviral properties (Baradaran-Rafii, Aghayan, Arjmand, & Javadi, 2007). Also, studies have shown interferon (IFN) synthesis and secretion by amniotic epithelial cells in quantities similar to immune leukocytes and skin fibroblasts with substantial anti-viral effects (Ferreira, de la Rogue, Rumjanek, & Golgher, 1992).

AME cells have been shown to inhibit proteinase activity, and inhibit infiltration and chemotactic activity of polymorphonuclear leukocytes and macrophages (Hori, Wang, Kamiya, Takahashi, & Sakuragawa, 2006). AME cells have also been shown to reduce proliferation of T and B cells, and suppress interleukin activity of IL-1α and IL-1β (Hori, Wang, Kamiya, Takahashi, & Sakuragawa, 2006). However, AME cells can be induced to express a pro-inflammatory molecule ICAM-1, a facilitating leukocyte adhesion and homing molecule and thus plays an important role in inflammation, by TNF-α and IL-1β (Dua, Gomes, King, & Maharajan, 2004). The stroma of the AM has been shown to effectively suppress inflammation by the inhibition of IL-1α and IL-1β (Fernandes, Mittanamalli, Sangwan, & Rao, 2005). Also, the secretion of TIMP-1, -2, -3, and -4 by AME cells further supports an anti-inflammatory role/property of the AM (Baradaran-Rafii, Aghayan, Arjmand, & Javadi, 2007).
Initially, it was believed that amniotic epithelia cells did not express Human Leukocyte Antigen (HLA) molecules because amniotic membrane grafts were not rejected post transplantation (Hori, Wang, Kamiya, Takahashi, & Sakuragawa, 2006; Dua, Gomes, King, & Maharajan, 2004). This includes the lack of expression of HLA-A, -B, -D, and -DR antigens or β2-microglobulin on their cell surface (Hori, Wang, Kamiya, Takahashi, & Sakuragawa, 2006; Dua, Gomes, King, & Maharajan, 2004). Although initial studies using AM as transplant grafts suggested that AME cells did not express HLA molecules, it was shown that these HLA class I molecules were synthesized within AME (Adinolfi, et al., 1982). Following, a study found strong expression of HLA class I molecules in AME cells, including cells of the AM mesenchyme but no expression of HLA class II molecule (Kubo, Sonoda, Muramatsu, & Usui, 2001). HLA class Ib has been shown to be expressed on term AME cells with very little expression of HLA class Ia molecules (Dua, Gomes, King, & Maharajan, 2004). Also, it was shown that HLA class II molecules are expressed by amniotic mesenchymal macrophages with a large increase in expression with an increase in gestational age (Dua, Gomes, King, & Maharajan, 2004). More thorough investigations of HLA molecules expression or absence of such is suggested, which would increase and better our understanding of the low to no immunogenic properties of the AM.

Also, AME cells have shown express the three complement factors: CD59, decay-accelerating factor (DAF), and membrane attack complex (MAC) inhibitory protein (MIP) (Kubo, Sonoda, Muramatsu, & Usui, 2001). CD59 and DAF are
known to protect cells against lysis by complement (Kubo, Sonoda, Muramatsu, & Usui, 2001). Expression of these factors on AME cells is suggestive that it is protective measures against maternal complement factors (Kubo, Sonoda, Muramatsu, & Usui, 2001).

2.5.3 Promoter of Epithelialization

The AM promotes epithelialization through two mechanisms, which are not mutually exclusive (Baradaran-Rafii, Aghayan, Arjmand, & Javadi, 2007). The first is the AM serving as a BM scaffold in which epithelial cells can migrate successfully (Dua, Gomes, King, & Maharajan, 2004). This AM scaffold reinforces adhesion of basal epithelial cells, promotes epithelial differentiation, and prevents epithelial apoptosis (Dua, Gomes, King, & Maharajan, 2004). The second means is by paracrine actions (Dua, Gomes, King, & Maharajan, 2004). In studies showing AM promotion of epithelialization, cytological and molecular assays show AME cells secreting EFG, TGF-α, KGF, HGF, b-FGF, TGF-β1, -2, -3, and the expression of growth factor receptors including KGFR and HGFR by such cells (Dua, Gomes, King, & Maharajan, 2004). These factors and receptors play vital roles in epithelialization, and the expression of such suggest this very phenomenon (Dua, Gomes, King, & Maharajan, 2004).

2.5.4 Inhibitor of Angiogenesis

The AM has been shown to inhibit endothelial cell growth, inhibit neovascularization of the cornea, and decrease vascularization of the ocular
surface (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012). These studies suggest an overall anti-angiogenic property of the AM (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012). It is suggested that two factors are at work that promote such an anti-angiogenic effect of the AM (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012). The first is the physical barrier the AM provides, in which it acts by preventing diffusion of growth and migratory factors necessary for vascularization, specifically endothelial and vascular cell growth and migration (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012). Also, the AM has been shown to contain proteins involved in suppressing angiogenesis, and produce various anti-angiogenic cytokines (Mamede, Carvalho, Abrantes, Laranjo, Maia, & Botelho, 2012). Proteins believed to suppress angiogenesis that can be found in the AM include collagen types IV and VII, laminin-1, and -5, and fibronectin; and anti-angiogenic factors include endostatin, thrombospondin-1, which was shown to be expressed by AME cells but not found in the AM mesenchymal stroma (Fernandes, Mittanamalli, Sangwan, & Rao, 2005). The protein pigment epithelium-derived factor (PEDF) is expressed by the AM, and is known to suppress endothelial cell migration and proliferation, and retinal neovascularization (Dua, Gomes, King, & Maharajan, 2004).
2.5.5 Activator of Apoptosis

The AM induces cell death or apoptosis in various cell types (Hori, Wang, Kamiya, Takahashi, & Sakuragawa, 2006; Li, He, Kawakita, Espana, & Tseng, 2006). As discussed above, the AM suppresses the immune response through various cytokine factors, it is these factors that suppress and induce death in cells of the opposing, pro-inflammatory response (Hori, Wang, Kamiya, Takahashi, & Sakuragawa, 2006; Dua, Gomes, King, & Maharajan, 2004; John, 2003). The AM has been shown to induce apoptosis in inflammatory macrophages through cell signaling effects including down-regulating NF-κB and Akt-FKHR signaling pathways (Li, He, Kawakita, Espana, & Tseng, 2006). AM transplants have also been shown to induce apoptosis in T lymphocytes, further down regulating the pro-inflammatory response, particularly T lymphocyte subsets responsible for macrophage type 1 cells (Hori, Wang, Kamiya, Takahashi, & Sakuragawa, 2006; Li, He, Kawakita, Espana, & Tseng, 2006).

2.5.6 Anti-Fibrotic/Inhibitor of Scarring

AME has been shown to inhibit scar formation and fibrosis by suppressing the activity of TGF-β, and TGF-β receptors found on AM stromal fibroblasts, inhibit fibroblast differentiation, which is induced by TGF-β (Hori, Wang, Kamiya, Takahashi, & Sakuragawa, 2006). Also, AM inhibits keratocyte to myofibroblast differentiation (Hori, Wang, Kamiya, Takahashi, & Sakuragawa, 2006). The myofibroblast cell is important in the latter stages of fibrosis and scarring.
necessary for wound contraction (Baradaran-Rafii, Aghayan, Arjmand, & Javadi, 2007).

2.5.7. Anti-Carcinogenic

AM-MSC have been shown to significantly reduce the proliferation of cancer cells of hematopoietic and non-hematopoietic cell lines (Magatti, De Munari, Vertua, & Parolini, 2012). Proliferation reduction via cell-cell contact and through unknown soluble factors arrests the cell cycle of these cancer cells in the G0/G1 phase (Magatti, De Munari, Vertua, & Parolini, 2012).
CHAPTER 3

AMNIOTIC FLUID

3.1 Historical Perspectives

Scientific knowledge on AF was merely non-existent before the 1900s (Longo & Reynolds, 2010). Much knowledge on AF before the 20th century is immersed in mysticism, folklore, and superstitions, as was knowledge on the AM, which was discussed above in chapter 2 (Longo & Reynolds, 2010; Trelford & Trelford-Sauder, 1979). For millennia it had been known that the AF bathes the developing embryo and fetus (Longo & Reynolds, 2010). This knowledge, supplemented with the many mystical and religious beliefs associated with life and creation, established the many beliefs associated with the AF during this period (Longo & Reynolds, 2010). Referencing the amniotic fluid are the ancient Hindu scriptures the Upanishads, ancient Buddhist scriptures and scared texts, including the Christian Bible, and the holy Qu’ran of the Islamic faith (Carr, 2004; Elsaie, 2004; Olivelle, 1998; Shakya, 1998). These beliefs, although scientifically inaccurate, however, were nonetheless important in defining the vital and necessary roles of the AF in the creation and development of a new life (Longo & Reynolds, 2010). Below is a description of these many beliefs of the amniotic fluid prior to the 20th century. Following is a description of the scientific knowledge accrued on the AF at the turn of the 20th century.
3.1.1 Pre-20th Century

3.1.1.1 Vedic Period (1700BCE–500BCE)

Of all religious texts that make such references to the AF it is the Upanishads that are of the oldest (Olivelle, 1998). As mentioned above in Chapter 2, the Upanishads are philosophical texts considered to be the core of the Hindu religion (Olivelle, 1998). Referencing the AF is one of the thirteen principle Upanishads, Chāndoya Upanishad (Olivelle, 1998). The Chāndoya Upanishad, “you are that”, seeks to explain the origin of the universe (Olivelle, 1998). Interestingly, in the Chāndoya Upanishad we are told that the universe developed from an egg that split in two upon hatching (Olivelle, 1998). Even more interesting, was the reference of two membranes of the egg, which have figurative representation, the outer membrane being the mountains, and the inner membrane, the AM, being the clouds and mist, mentioned above (Olivelle, 1998). Within the membranes is a fluid, AF, which represents the nourishing mother ocean (Olivelle, 1998).

3.1.1.2 Ancient Greece (1200BCE–300BCE)

Aristotle was the first to describe AF stained with meconium, which was associated with fetal death at the time (Iliodromiti, Grigoriadis, Vrachnis, Siristatidis, Varras, & Creatsas, 2012). The term meconium meaning “opium-like” is believed to derive from the color and appearance of AF stained with
meconium\textsuperscript{84}. Aristotle described such an appearance of being black with a thick consistency very similar to processed opium (Iliodromiti, Grigoriadis, Vrachnis, Siristatidis, Varras, & Creatsas, 2012). This is one of the only mentions of AF in existing ancient Greek scientific texts (Iliodromiti, Grigoriadis, Vrachnis, Siristatidis, Varras, & Creatsas, 2012; Longo & Reynolds, 2010).

3.1.1.3 Africa (700BCE-present)

The Yorùbá culture dates back to the 7\textsuperscript{th} century BCE, and entails people sharing common and ethnicity originating from the Southwestern region of Nigeria (Zenon, 2011). The Yorùbá culture and religion encompasses a vast array of rituals, prayers, stories, and spiritual beliefs (Zenon, 2011). These religious practices have become part of the many Caribbean, Middle American, and South American cultures of the West Indies for a vast majority of Africans sold in the slave trade were of Yorùbá decent (Zenon, 2011). The African Yorùbá culture places heavy significance in the AF in fertility and growth of a fetus (Zenon, 2011). One particular belief of the Yoruba culture is the power harnessed by the goddess and divine force, Yemoja (Zenon, 2011). Yemoja is believed to be the mother of waters, giving rise to all natural water resources, including the ocean and AF (Zenon, 2011). It is her influence and forces through the AF that drive the creation and sustainability of the life’s new creation, and the protection from death and disease (Zenon, 2011).
3.1.1.4 South Asia (500BCE-present)

In Buddhism, Guanyin, a Bodhisattva of compassion, is depicted to be holding a willow branch in one hand and a flask that contains and pours the “Dew of Immortality” (Shakya, 1998). This Dew or fluid within the flask of which Guanyin holds is a nutritive broth or milk of elixir properties often thought to be equivalent to AF (Shakya, 1998). In Buddhist beliefs, AF was considered to be the nutritious broth that fed the fetus, which today is scientifically accurate (Shakya, 1998).

3.1.1.5 Ancient Rome (500BCE-400AD)

The Christian belief that one must be born again in order to be saved is stated in the bible, “Truly, truly, I say to you, unless one is born again, he cannot see the kingdom of God” (John 3:3) (Carr, 2004). The various Christian denominations have different interpretations of this quote (Carr, 2004). Many denominations, including Catholicism, believe being born again is the simple act of baptism (Carr, 2004). Others, specifically those of the Evangelical and Calvinist denominations, believe that the water being referenced is not baptism water but AF, and thus one must be born within the fluid of the AM to be born again (Carr, 2004). Thus, the very act of being born indicates a rebirth of a soul that will ascend to the Gates of Heaven upon their calling (Carr, 2004).
3.1.1.6 Western Africa (1100AD-present)

The Akan culture is a matrilineal culture that entails the current ethnic groups of Africa’s Ghana and Cote d’Ivoire (Akhan, 2010). The origin of this culture dates back many centuries; however, the first firm establishment of this culture dates back to the 12th century (Akhan, 2010). The beliefs of this culture center on the universal governing of spirit-forces, Abosom (Akhan, 2010). These forces control creation, and all celestial bodies and matter of the universe (Akhan, 2010). According to Akan beliefs, energy of a nurturing mother was equivalent to energy of the moon or bosom (Akhan, 2010). It was believed that the AF within the womb of the mother was physically and spiritually equivalent to Earth’s oceans (Akhan, 2010). The salt of the ocean was that of God’s tears, a means of ablution created to cleanse and purify the souls of the people (Akhan, 2010). The fluid of the womb, AF, served to hold a new creation in a state of purity for it was believed that the salt within keeps the fetus in such a state (Akhan, 2010). Also, according to Akan beliefs, it was the mother that had true influence on the spirits and physiology of the embryo and fetus (Akhan, 2010). It was assumed that the mother’s influence over the fetus was equivalent to the moon’s influence on the oceans of earth; bosom’s huge effect and influence on the rising and falling of the ocean’s tide on earth, so does the mother on her fetus by effecting the tides of the AF (Akhan, 2010). Thus, it is believed by the Akan culture that the AF is a
conductor of moods and emotion, and thus has not only an impact or effect on physiology of the developing fetus but also an effect on the spirits of the child (Akhan, 2010). These beliefs regarding the AF are still held among existing Akan people throughout the world (Akhan, 2010).

3.1.1.7 Early Modern Period (1450AD-1750AD)

In the 16th century Realdo Columbus, an Italian anatomist and surgeon, refuted the Hippocratic idea of “uterine paps” as the source of fetal nourishment (Power & Schulkin, 2012). Realdo Columbus correctly concluded that the fetus was protected by AF, but incorrectly postulated that this AF was the product of fetal sweat (Power & Schulkin, 2012).

Also, in the 17th century The English physician William Harvey believed that the developing fetus received its nourishment from the AF by swallowing it, and by diffusion through pores in the skin of the fetus (Longo & Reynolds, 2010). Nicolas van Hoboken a Dutch anatomist and physician, through the study of cow fetuses, believed nourishment of the fetus was also through the AF (Longo & Reynolds, 2010). However, he incorrectly postulated that small pores in the CM, which allowed passage of molecules from the uterus or maternal blood circulation, were the derivation of such nourishment of the AF (Longo & Reynolds, 2010). He also believed the CM and AM were not vascularized, and thus no maternal vascularization of the CM, which has been shown to be incorrect (Longo & Reynolds, 2010).
3.1.2 Post-20th Century

3.1.2.1 Advent of the Amniocentesis (1870s)

The scientific study of AF at the turn of the 20th century can be most attributed to the advent of the amniocentesis (Magana & Shah, 2013; Uyeno, 1919). Amniocentesis is the withdrawal of AF from the amniotic sac using a long, fine needle (Magana & Shah, 2013). German physicians Prochownick, Von Schatz and Lambi performed the first documented amniocentesis in the 1877 to relieve pressure on a fetus suffering from hydramnios (Magana & Shah, 2013; Uyeno, 1919). Following, Dr. Prochownick analyzed AF obtained from amniocentesis for sodium chloride, solid substances, and urea content (Uyeno, 1919). And in 1891, Dr. Schroeder studied the content of solid substances, ash, and albumin in AF samples obtained from amniocentesis (Uyeno, 1919). It is uncertain if these studies conducted by Drs. Prochownick and Schroeder were conducted on human AF samples (Magana & Shah, 2013; Uyeno, 1919).

3.1.2.2 Indication of Amniotic Fluid Cells (1950s-1970s)

The first documentation in which the cellular composition of AF was analyzed can be attributed to the obstetrician Dr. Fritz Friedrich Fuchs and gastroenterologist Polv Riis (Magana & Shah, 2013). In 1956 they showed that fetal sex could be determined from AF cells taken from an amniocentesis by identifying the presence or absence of Barr bodies within these fetal cells.
(Magana & Shah, 2013). After, the further characterization of AF cellular and non-cellular contents would ensue (Magana & Shah, 2013).

Widespread prenatal screening introduced itself in the late 1960s wishing to both increase research in reproductive health and detect pregnancy as early as possible (Nadler, 1969). Routine screening of the fetus and AF brought about a heightened investigation of the cells within the AF (Nadler, 1969). Research on AF cells at the time was extremely limited often only including the culturing of such cells for morphological identification and genetic karyotyping (Nelson & Emery, 1973). In 1966 the first documented, successful culture of fetal AF cells for genetic screening was done by Dr. Steele and Dr. Breg (Kelley, 2013). The culture of fetal AF by Steele and Breg revealed two distinct morphological classes of cells: epithelial-like and fibroblast-like cells (Kelley, 2013). It was in 1969, in the lab of Dr. Nelson and Emery of Edinburgh, Scotland, in which research in to AF cell culture modification and optimum growth settings occurred (Nelson & Emery, 1973). Here, it was first shown that AF cells adhered to glass surfaces promoting cell growth in culture (Nelson & Emery, 1973). It was concluded that successful, viable amniotic fluid cell growth occurred most significantly in AF obtained less than 20 weeks of gestation (Nelson & Emery, 1973). Over the course of the next two decades, culture techniques would drastically improve, increasing both the acceleration of growth and viability of AF cells (Chang, Jones, & Masui, 1982; Martin, 1980; Porreco, Bradshaw, Sarkar, & Jones, 1980; Rudiger, Wolff, Wendel, & Passarge, 1974). Together with
improved growth conditions and advancement in cytological and biochemical technologies, further identification and understanding of AF cells and non-cellular composition of such fluid would ensue (Kelley, 2013; Chang, Jones, & Masui, 1982; Martin, 1980; Rudiger, Wolff, Wendel, & Passarge, 1974).

In 1971, the two principle cell types of AF cell culture initially demonstrated by Steele and Breg (above) were shown to be not only morphologically distinct but also biochemically distinct by opposing histidase activity in both cell class types (Melancon, Lee, & Nadler, 1971). Epithelial-like cells were shown to have histidase activity but fibroblast-like cells lacked histidase activity (Melancon, Lee, & Nadler, 1971). Hoehn et al. conducted further investigation on principle amniotic fluid cell types in 1974 (Hoehn, Bryant, Karp, & Martin, 1975). A large sample size of second trimester amniotic fluid cell cultivation revealed three main classes of amniotic fluid cells with corresponding proportions: 5.5% fibroblast-like cells (F-type cells), 33.7% epitheloid (E cells), and 60.8% previously identified AF specific cells (Hoehn, Bryant, Karp, & Martin, 1975).

Following, in 1974 Sutherland et al. cultured AF specimens of second trimester pregnancies (Sutherland, Bauld, & Bain, 1974). Throughout the course of cultured samples, an inverted microscope was used to characterize cells based on morphology (Sutherland, Bauld, & Bain, 1974). Five cell types were readily identifiable and characterized throughout the study: macrophage cells, three types of E cells, and a F-Type cell (Sutherland, Bauld, & Bain, 1974).
In 1982 a study revealed the presence of goblet cells, urotheial cells, histiocytes, macrophages, AME cells, and umbilical cord cells in natural AF (Schrage, Bogelspacher, & Wurster, 1982). Unfortunately, the vast majority of cells in natural AF are washed off during the culture process, and thus explains why information on all AF cells is lacking, even today (Prusa & Hengstschlager, Amniotic fluid cells and human stem cell research - a new connection., 2002). It is believed that a large part of the washed off, non-adhering cells in culture are exfoliated squamous cells, including identified goblet cells, histiocytes, and macrophages (Prusa & Hengstschlager, Amniotic fluid cells and human stem cell research - a new connection., 2002; Schrage, Bogelspacher, & Wurster, 1982).

With the increasing use of AF cells for prenatal diagnosis, the urge to identify congenital pathologies unidentifiable through karyotyping and cellular morphology brought about the identification of properties of AF cells previously unknown (Pollack, Heagney, Braun Jr., & O'Neill, 1981; Rosenmann, Schumert, Theodor, Cohen, & Brautbar, 1980). In 1980 the use of AF cells to identify and diagnose in utero congenital adrenal hyperplasia due to 21-hydroxylase deficiency and complement C4 deficiency, pathologies both known to be heavily linked to HLA expression, showed the expression of HLA antigens on amniotic fluid cells, particularly HLA-B homozygosity (Pollack, Heagney, Braun Jr., & O'Neill, 1981; Rosenmann, Schumert, Theodor, Cohen, & Brautbar, 1980). Further testing revealed the strong presence of HLA-A,-B, and –C antigens in AF cells with the absence of HLA class II molecules, particularly HLA-D class II.
molecules – as shown above, indirectly (Pollack, Heagney, Braun Jr., & O'Neill, 1981; Rosenmann, Schumert, Theodor, Cohen, & Brautbar, 1980; Valentine-Thon, Kreeb, Grosse-Wilde, & Passarge, 1983). However, in 1984 Kreeb et al. found that a small subpopulation of cultured AF cells expressed HLA-DR class II molecules using monoclonal cDR antibodies (Kreeb, Valentine-Thon, Krumbacher, Grosse-Wilde, & Passarge, 1984). Such a discrepancy can be possibly attributed to the arbitrary definition of what is considered positive and negative for HLA class I and II. HLA class I molecules deemed negative in AF cell cultures in the study conducted by Zhang et al. was less than 2% positive (Kreeb, Valentine-Thon, Krumbacher, Grosse-Wilde, & Passarge, 1984; Valentine-Thon, Kreeb, Grosse-Wilde, & Passarge, 1983). The small population positive for HLA class II molecules in Kreeb et al. could possibly represent the small 2% found in the previous study (Kreeb, Valentine-Thon, Krumbacher, Grosse-Wilde, & Passarge, 1984). Future aspects of HLA typing would be used in research investigation of AF cells and stem cells to confirm fetal origin of such cells, and later confirmed to be responsible for low-immunogenicity of AF-derived stem cells (Kreeb, Valentine-Thon, Krumbacher, Grosse-Wilde, & Passarge, 1984). Following, future testing of AF to identify congenital pathologies would be evaluated for a myriad of disorders (Aula, et al., 1980; Gosden & Brock, 1978; Gosden & Brock, Morphology of rapidly adhering amniotic-fluid cells as an aid to the diagnosis of neural-tube defects., 1977; Sutherland, Bauld, & Bain, 1974).
3.1.2.3 Identification of Amniotic Fluid Stem Cells (1990s-present)

The presence of cells in first trimester AF remained a mystery until 1993 (Fauza, 2004). In 1993 Torricelli et al. cultured AF samples from the 7th to 13th weeks of gestation obtained from voluntary abortions (Fauza, 2004). Cells were identified in culture of AF samples of gestational weeks equal to or greater than 11 weeks (Fauza, 2004). The morphology of identified cells in the 11th week of gestation included small nucleated, round cells, most consistent with hematopoietic progenitor cells (Fauza, 2004). The identification of progenitor cells in AF by this study was the first ever to signify a possible presence of stem cells in AF (Fauza, 2004).

In 2001, Kaviani et al. mechanically isolated a subpopulation of cells of second trimester AF samples positive for smooth muscle actin, vimentin, cytokeratin 18, and fibroblast surface protein most consistent with a mesenchymal, fibroblast-myofibroblast cell lineage (Kaviani, Perry, Dzakovic, Jennings, Ziegler, & Fauza, 2001). After isolation, these cells were rapidly expanded in culture, and seeded on to an acellular human dermis scaffold (Kaviani, Perry, Dzakovic, Jennings, Ziegler, & Fauza, 2001). These cells firmly adhered to the decellularized scaffold with increased density and with no evidence of cell death (Kaviani, Perry, Dzakovic, Jennings, Ziegler, & Fauza, 2001). This experiment simply showed therapeutic potentials of AF cells,
strengthening the possible presence of stem cells within AF (Kaviani, Perry, Dzakovic, Jennings, Ziegler, & Fauza, 2001).

Following, in 2003 Prusa et al. characterized a subpopulation of AF cells that expressed the transcription factor Oct-4, a “stemness” factor found in embryonic stem cells (Prusa, Marton, Rosner, Bernaschek, & Hengstschlager, 2003). And in 2004 AMSC were isolated, cultured, expanded, and confirmed of fetal origin using HLA typing (Tsai, Lee, Chang, & Hwang, 2004). These cells were isolated based on cell morphology consistent with AFMSC above (Tsai, Lee, Chang, & Hwang, 2004). They showed multilineage differentiated potentials into fibroblasts, adipocytes, and osteoblasts (Tsai, Lee, Chang, & Hwang, 2004). Also, these cells showed a small subpopulation of Oct-4 (Tsai, Lee, Chang, & Hwang, 2004).

In 2007 De Coppi showed that about 1% of AF cells expressed the stem cell factor receptor CD-117, or c-kit (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012). These cells were shown to differentiate in to all three germ layers (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012). It was this study that highly emphasized a population of AFDSC distinct from the originally identified AMSC (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012; Tsai, Lee, Chang, & Hwang, 2004; Kaviani, Perry, Dzakovic, Jennings, Ziegler, & Fauza, 2001). What is uncertain is if these cells are truly representing an absolute heterogeneous stem cell clonal population. It has also brought about a
convoluted nomenclature of stem cells in AF, supported by a lack of information regarding their origin, and a non-concomitant expression of stem cell cytological and biochemical markers (Fauza, 2004). To be clear, amniotic fluid-derived stem cells (AFDSC) represent all stem cells found in AF, and amniotic fluid mesenchymal stem cells (AMSC) represent stem cells isolated by Kaviani et al. in 2001 and Tsai et al. 2004 (Tsai, Lee, Chang, & Hwang, 2004; Kaviani, Perry, Dzakovic, Jennings, Ziegler, & Fauza, 2001). Stem cell subpopulations found in AF said be distinct from AMSC, including CD117+ stem cells fall under AFDSC (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012).

In 2013, Ryan et al. investigated the expression of Oct-4 in fetal and placental mesenchymal stem cells (Ryan, Pettit, Guillot, Chan, & Fisk, 2013). Familiar with the major role of Oct-4 in embryonic stem cell but equipped with the knowledge of Oct-4 gene variants, pseudogenes, and isoform complexities, Ryan et al. concluded that studies identifying Oct-4 positive cell populations as stem cells were falsely led; and thus ultimately questioning the “stem-ness” of Oct-4 positive stem cells in AF (Ryan, Pettit, Guillot, Chan, & Fisk, 2013). It should be known that identification of isolated AMSC, initially and current, for “stem-ness” was and is done using various characteristics, including cell morphology and cytological markers (Tsai, Lee, Chang, & Hwang, 2004; Kaviani, Perry, Dzakovic, Jennings, Ziegler, & Fauza, 2001). Oct-4 identification in AMSC was used for additional identification but never primary verification of “stem-ness” as were CD117+ cells (Ryan, Pettit, Guillot, Chan, & Fisk, 2013; Cananzi & De Coppi,
CD117+ amniotic fluid cells., 2012; Kaviani, Perry, Dzakovic, Jennings, Ziegler, & Fauza, 2001; Tsai, Lee, Chang, & Hwang, 2004).

3.2 Volume and Regulation

The volume and regulation of AF throughout gestation are functions of embryonic and extraembryonic membranes (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005). AF volume is influenced and regulated by six water and solute pathways each having a certain level of influence during normal and pathological states (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005; Brace, Physiology of amniotic fluid and volume regulation., 1997). Derivation of the AF is highly dynamic and is more than simply the diffusion of maternal plasma into the developing amniotic cavity (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005; Brace, Physiology of amniotic fluid and volume regulation., 1997). The state of volume of AF regulation is highly complex for it consists of the simultaneous occurrence of water and solute exchange among six pathways (Brace, Physiology of amniotic fluid and volume regulation., 1997).

Early in gestation newly established amnioblasts form the membrane of the newly forming amniotic cavity within the primary ectoderm on day 8 post-fertilization (Larsen, 1997). Existing between these amnioblasts and throughout gestation are intercellular tight junctions with varying permeability (Larsen, 1997). At the very beginning of amniotic sac development water of the maternal plasma
circulating in the establishing trophoblast is able to diffuse into the primordial amnion and thus establish its existence (Larsen, 1997). Throughout gestation these cells will function in the transfer of amniotic fluid between maternal circulation and the amnion fluid (Larsen, 1997).

3.2.1 Intramembranous Pathway

The first pathway is the intramembranous pathway, which is the exchange of fluid and solutes between AF and fetal blood perfusing fetal surface of the placenta, fetal skin, and the umbilical cord (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). In humans, the primary intramembranous pathway exchange occurs across the fetal surface of the placenta and the fetal vessels of the chorionic plate (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). This pathway is believed to play a major role in fluid volume and composition regulation in early gestation until full-keratinization of fetal skin 22 weeks gestation from then of which it is thought to play a small, insignificant role in contribution and regulation of AF volume and composition (Brace, Physiology of amniotic fluid and volume regulation., 1997; Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). Although minor in its role in later stage gestation in AF volume and composition, the
intramembranous pathway nutrient absorption does occur and is a function of para-cellular pathways and fetal blood stream permeability, indicating an influence regulated by hormones and molecules (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). In a near term fetus 200-500 ml of fluid is absorbed by the intramembranous pathway (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). It is believed that AF volume and regulation is controlled by numerous factors including trans-cellular vesicular transport mechanisms and the possibility of aquaporin proteins in fetal membranes, and hormone regulation including decidual prolactin thought to increase fetal membrane permeability (Brace, Physiology of amniotic fluid and volume regulation., 1997; Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995).

3.2.2 Transmembranous Pathway

Exchange between maternal membrane of the CM and of the fetal AM is the transmembranous pathway (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). This pathway is believed to play only a small role in AF volume in normal states throughout gestation, being most significant during initial
development of the amniotic cavity mentioned above (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). However, it has been shown that this pathway plays a significant role in regulating AF volume during times of maternal dehydration as shown by the increase in AF volume through this pathway during extreme maternal dehydration and fetal oligohydramnios; however, insignificant during normal state perturbations (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). It is believed that this pathway is highly regulated by maternal aldosterone and ADH (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005). Also, prostaglandin excreted by the fetal kidney and fetal lungs and also released by the AM and CM is thought to heavily influence the transmembranous pathway, possibly explaining an increased role in such during certain pathologies in which this hormone is increased (Sherer, 2002). During later stages of gestation the transmembranous pathway contributes 0.3% of volume of fetal body weight, with flow of water in latter stages to be outward to be about 10 ml of amniotic fluid (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995).
3.2.3 Fetal Urine Pathway

The third pathway is that of fetal urine excretion by the fetus (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). Amniotic fluid volume in the second half of gestation is in large part due to the fetal urine pathway (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). Fetal urine first enters the amniotic sac at 8-11 weeks of gestation (Modena & Fieni, 2004). At about 25 weeks of gestation urine excretion is about 110 ml/kg for every 24-hour period; and at about 39 weeks of gestation urine excretion is about 190 ml/kg for every 24-hour period (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). At term, urine excretion is about 800-1200 ml/day (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995).

3.2.4 Fetal Respiratory Secretion Pathway

The fourth through sixth pathways include respiratory and oral-nasal secretions, and fetal swallowing (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005; Brace, Physiology of amniotic fluid and volume regulation., 1997). These pathways play a small role in amniotic fluid volume and composition when compared to the first three pathways (Underwood,
Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005; Brace, Physiology of amniotic fluid and volume regulation., 1997). Not until recently did the idea of fetal lung secreted fluid in to the amniotic fluid become accepted (Brace, Physiology of amniotic fluid and volume regulation., 1997). Prior, fetal lung absorption was believed to be the main courses of action of which the fetal lungs endured, however, it has been elucidated that only under fetal asphyxia or severe distress do fetal lungs absorb amniotic fluid (Brace, Physiology of amniotic fluid and volume regulation., 1997). Normal fetal lung secretion, out flow from the trachea occurs under normal fetal episodes of breathing, half entering the amniotic fluid and roughly half being swallowed (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). For a near term fetus 340 ml of fluid is secreted from the lungs, 170 ml in to the amniotic fluid and 170 ml is swallowed (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995).

**3.2.5 Fetal Oral-Nasal Secretions Pathway**

Oral secretions contribute only about 25 ml in to the amniotic fluid (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). Fetal oral-nasal secretions make small contributions to amniotic fluid volume and only during the latter stages of gestation (Underwood, Gilbert, & Sherman, Amniotic fluid: not just
fetal urine anymore., 2005; Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995).

3.2.6 Fetal Swallowing Pathway

Fetal swallowing is believed to play a significant role in amniotic fluid volume in the second half of gestation (Modena & Fieni, 2004). The fetus begins to swallow amniotic fluid at about the same time fetal urine excretion occurs, at about 8-11 weeks of gestation; with about 17 ml of amniotic fluid being swallowed in early gestation (Modena & Fieni, 2004). This volume does not include respiratory secretions swallowed before excreted into the amniotic sac (Modena & Fieni, 2004). Animal studies have shown that prevention of fetal swallowing, fetal urine output remained normal and amniotic fluid volume was no different than controls (Gilbert & Brace, 1993). In near term fetuses about 500-1000 ml of amniotic fluid is swallowed (Brace, Physiology of amniotic fluid and volume regulation., 1997; Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995).

Overall, there is about 30 ml of amniotic fluid in an amniotic sac of 10 weeks gestation and 190 ml at 16 weeks of gestation (Modena & Fieni, 2004). At the beginning of gestation until about fetal keratinization occurs amniotic fluid volume is a multiple of fetal volume with both volumes becoming equal at about
the 21st to 22nd week of gestation (Modena & Fieni, 2004). It should be known that during the last trimester there is a net increase of 30 to 40 ml of amniotic fluid volume per day (Modena & Fieni, 2004). Around the 30th week of gestation amniotic fluid volume is about half the volume of the growing fetus (Modena & Fieni, 2004). Also, at this time amniotic fluid volume is around 780-800 ml (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005; Modena & Fieni, 2004). After 32-35 weeks amniotic fluid volume beings to decrease and at term the amniotic fluid volume is about one-quarter that of fetal volume with about 500-600 ml of amniotic fluid can be found in the amniotic sac of a term fetus (Modena & Fieni, 2004).

The roles of fetal and extraembryonic membranes in volume and regulation are still poorly understood (Modena & Fieni, 2004; Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). What is most confusing is the discrepancy one finds when comparing net amniotic fluid volume movement when compared to diffusion water factors of the intramembranous and transmembranous pathways (Brace, Progress toward understanding the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995). It is for this reason that studies and information that provide insight into water movement among the aforementioned pathways provide only a small amount of information in the regulation of amniotic fluid when pathways are examined holistically (Brace, Progress toward understanding
the regulation of amniotic fluid volume: water and solute fluxes in and through the fetal membranes., 1995).

3.3 Non-Cellular Composition

The non-cellular composition of amniotic fluid include inorganic electrolytes, amino acids, peptides, and proteins, lipids, carbohydrates, growth factors, urea, and cytokines\textsuperscript{118}. The composition of these molecules is a function of the embryonic and extraembryonic membranes, and thus changes throughout pregnancy (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005). Unfortunately, discussion of these molecules below does not address how they change as a function of gestational time but only includes their absolute presence throughout gestation.

3.3.1 Inorganic Molecules

Sodium (Na\textsuperscript{+}) and Chloride (Cl\textsuperscript{−}) are the major inorganic electrolytes found in amniotic fluid (Uyeno, 1919). Their concentration within the amniotic fluid ranges from about 0.444% to 0.58% of amniotic fluid non-cellular composition (Uyeno, 1919). Other inorganic molecules found in the amniotic fluid but in trace amounts include K+, Ca\textsuperscript{2+}, Li+, Rb\textsuperscript{+}, Mg\textsuperscript{2+}, Sr\textsuperscript{2+}, and Ba\textsuperscript{2+} (Palavinskas, Kriesten, & Schulten, 1983).
3.3.2 Amino Acids and Carbohydrates

Prior to fetal skin keratinization, amino acids freely diffuse from the amniotic fluid from maternal absorption to fetal circulation (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005). However, after keratinization of the fetal epidermis, amino acids obtained from the amniotic membrane are unable to free cross into the fetus, and thus concentration is increased in amniotic fluid (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005). Also, amino acids are excreted into the amniotic fluid through the fetal urine pathway (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005). Amniotic fluid is rich in the amino acid taurine, which is higher in concentration relative to fetal and maternal serum, which is the opposite for other amino acids (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005). Other amino acids found in amniotic fluid include but are not limited to glutamine and arginine (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005).

Carbohydrates and their derivatives found in the amniotic fluid include glucose, fructose, lactate, and pyruvate (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005).

3.3.3 Proteins and Peptides

Proteins found in amniotic fluid include, fibronectin, creatinine, α-defensins, lactoferrin, lysozyme, bactericidal/permeability-increasing protein, calprotectin,

3.3.4 Growth Factors

Growth factors found in amniotic fluid include epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), transforming growth factor beta-1 (TGF-β1), insulin-like growth factor I (IGF-I), erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF) (Underwood, Gilbert, & Sherman, Amniotic fluid: not just fetal urine anymore., 2005).

3.3.5 Steroids

Steroids found in amniotic fluid include testosterone, androstenedione, dehydroepiandrosterone (DHA), DHA sulfate (DHAS), progesterone, 17α-hydroxyprogesterone (17-OHP), estradiol, and hydrocortisone (Carson, Okuno, Lee, Stetten, Didolkar, & Migeon, 1982).

3.3.6 Cytokines

Cytokines are found in small concentrations in amniotic fluid. These cytokines include IL-6, IL-8, TGF-β, TNF-α, IL-11, and IL-15. Cytokines found in very small concentrations in the amniotic fluid include IL-10 and IL-12 (Heikkinen, Mottonen, Pulkki, Lassila, & Alanen, 2001).
3.4 Cellular Composition

3.4.1 Non-Stem Cell

3.4.1.1 Cells of Uncultured Amniotic Fluid

Using EM two main groups of cells can be identified in uncultured, natural amniotic fluid based on ultrastructure: large cells with irregular borders and small nuclei, later defined as squamous cells; and small round macrophage-like cells (Gosden C. M., Amniotic fluid cell types and culture., 1983; Cutz & Conen, 1978). Another study confirmed the presence of large squamous cells with and without keratinization, and with regular microvilli borders (Cutz & Conen, 1978). The macrophage-like cells initially identified were confirmed in this study showing phagosomes in and phagocytosis in vitro abilities of the second, macrophage cells (Cutz & Conen, 1978). However, it has yet to be identified exactly what proportions of these two classes of cells account for the cells found in cultured amniotic fluid mentioned below (Gosden C. M., Amniotic fluid cell types and culture., 1983; Cutz & Conen, 1978).

3.4.1.2 Cells of Cultured Amniotic Fluid

Cells found in natural amniotic fluid can be classified into three broad groups based on cell culture characteristics: attaching, colony-forming amniotic fluid cells; attaching non-colony forming, non-proliferative amniotic fluid cells; and non-attaching amniotic fluid cells (Prusa & Hengstschlager, Amniotic fluid cells...
and human stem cell research - a new connection., 2002). The former group, attaching, colony-forming cells, has been significantly studied because of ease of cell culture growth, and is discussed below (Prusa & Hengstschlager, Amniotic fluid cells and human stem cell research - a new connection., 2002). Information and knowledge on the latter two groups is extremely limited (Prusa & Hengstschlager, Amniotic fluid cells and human stem cell research - a new connection., 2002). Also, it should be known that amniotic fluid cells identified in culture do not represent all cell types found in natural amniotic fluid (Prusa & Hengstschlager, Amniotic fluid cells and human stem cell research - a new connection., 2002).

3.4.1.3 Initial 3 Cell Classes of Cultured Amniotic Fluid

Cultured cell identification relies on morphological and culture growth characteristics (Hoehn, Bryant, Karp, & Martin, 1975). F-Type cells are spindle-shaped cells, grow in parallel arrays, and show high growth potentials (Hoehn, Bryant, Karp, & Martin, 1975). E cells show intimate cell-to-cell contact, resistant to trypsin detachment, and showed poor growth potential (Hoehn, Bryant, Karp, & Martin, 1975). The third group of cells are AF specific cells, which are indicated to be pleomorphic, and show intermediate growth potentials to those of F-type cells and E cells (Hoehn, Bryant, Karp, & Martin, 1975). These cells were briefly discussed above in the post-20th century historical perspectives section.
3.4.1.4 Five Cell Classes of Cultured Amniotic Fluid

Further investigation of epithelial-like cells in amniotic fluid including AF specific cells and E cells using intermediate filament antibodies, specifically keratin and vimentin antibodies, and the use of indirect immunofluorescence revealed the presence of five subtypes of E cells found in AF cell culture (Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981). Differentiated by their morphology, growth, and size E1 is the most common cell type in all cell cultures, pleomorphic in shape and size, fibroblast-like in morphology with bright fibrillar keratin-positive cytoplasms, showed no cell-to-cell interaction, and is also vimentin positive (Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981). E1 cell subtypes were previously classified as AF cells mentioned above (Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981). E2 cells show fibrillar organization of keratin and vimentin but lacked cell-to-cell organization of interaction, are large and flat in morphology, exhibited low growth and proliferation, and are present in all cell cultures but low in number (Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981). E3 cells are indistinguishable from E1 cells in size and morphology, but show keratin organization and cell-to-cell interaction, and diffuse vimentin-positive fibrils in the cytoplasm of the cells (Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981). E4 cells are classified by their rapid growth in culture, and show keratin organization and cell-to-cell interaction, and are present in a very small amount in culture (Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981). E5 cells are morphologically large, multinucleated cells dispersed among the other cell...
types in the AF culture (Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981). Also, E5 cells show bright locations of keratin fibers but also stain for vimentin antibodies (Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981). F-type cells are identified as vimentin-positive, keratin-negative cells (Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981). Cultures that show F-type cells as the dominant cell type in culture are very limited in number (Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981). Morphologically, E1, E3, and F-type cells are indistinguishable in culture, but can be differentiated by keratin and vimentin expression and cell-to-cell interaction (Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981). A positive immunofluorescence of keratin filaments in all cells except F-type cells, which includes both E cells and AF-specific type cells, strongly supports an epithelial origin of E cells and AF-specific cells (Chen, 1982; Virtanen, von Koskull, Lehto, Vartio, & Aula, 1981).

3.4.2 Stem Cells

Stem cell hallmark characteristics, or “stem-ness”, include self-renewal, multilineage differentiation, or pluripotency/multipotency; and maintain a state of dedifferentiation (Niwa, Miyazaki, & Smith, 2000). Identification of stem cells relies on the presence of transcription factors not expressed in differentiated cells, multilineage differentiation in culture in vitro, and telomerase activity (Niwa, Miyazaki, & Smith, 2000). A lack of one of the many properties does not exclude a cell from being that of a stem cell (Niwa, Miyazaki, & Smith, 2000). In fact,
Currently, the debate of an absolute definition defining “stem-ness” is ongoing, and entails a grey area in stem cell research (Niwa, Miyazaki, & Smith, 2000).

Principle transcription factors expressed in embryonic stem cells include Oct-4, NANOG, and Sox-2 all of which play a crucial role in embryonic stem cell self-renewal and pluripotency (Niwa, Miyazaki, & Smith, 2000). More specifically, Oct-4 is considered the master regulator of self-renewal and pluripotency in these cells (Pesce, Wang, Wolgemuth, & Scholer, 1998). During human embryonic development, Oct-4 is restricted to the inner cell mass of the blastocyst, and upon implantation and bilaminar disc formation it is restricted to the epiblast (Pesce, Wang, Wolgemuth, & Scholer, 1998). After gastrulation, Oct-4 expression is down-regulated in an anterior to posterior manner with expression found in germ cells thereafter (Pesce, Wang, Wolgemuth, & Scholer, 1998). In adult bone marrow mesenchymal stem cells not only express the transcription factors Oct-4, NANOG, and Sox-2 are expressed (Pesce, Wang, Wolgemuth, & Scholer, 1998). Also, transcription factors expressed in adult stem cells not expressed in embryonic stem cells believed to maintain their “stem-ness” include Rex-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (Pesce, Wang, Wolgemuth, & Scholer, 1998). Much of the research and data obtained from AFDSC including characteristics, properties, and potentials are from that of second semester amniotic fluid (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012; Da Sacco, De Filippo, & Perin, 2011).
Fibroblast-like cells isolated by Kaviani et al. have been shown to co-express CD44 and CD105 along with the expression of Oct-4 but negative for CD34 (Tsai, et al., 2007). Also, a more in-depth analysis of AMSC was conducted in conjunct with the analysis of bone marrow-derived stem cells (Nadri & Soleimani, 2007). AMSC were shown to be phenotypically similar to mesenchymal-derived stem cells from other tissues and progenitors, and differentiated cells of mesenchymal origin, further confirming their mesenchymal stem cell identity (Nadri & Soleimani, 2007; Tsai, Lee, Chang, & Hwang, 2004). Also, these cells not only express markers of mesodermal lineage but also express markers found on ectodermal and endodermal lineage cells, and given the appropriate differentiation media, are able to differentiate into all three germ layers, as discussed previously (Roubelakis, et al., 2007).

What distinguishes AMSC from adult mesenchymal stem cells, includes greater expansion potential, longer telomeres but no telomerase activity, and T-cell proliferation thus emphasizing its low-immunogenicity (In 't Anker, et al., 2003). When compared to induced pluripotent stem cells (iPS) and embryonic stem cells, AMSC have a stable karyotype; and do not display tumorogenic potentials when transplanted into immune-compromised mice, and bypass present ethical issues, respectively (Poloni, et al., 2011; Sessarego, et al., 2008). Another characteristic of AMSC that make them ideal stem cells for use in regenerative medicine and tissue engineering is their significant clonogenicity, or doubling in vitro (Poloni, et al., 2011; Sessarego, et al., 2008). Such a
characteristic highlights their remarkable ability of self-renewal over time with continued genetic stability not found in iPS or adult stem cells, and without tumor production, found in embryonic stem cell culturing (In 't Anker, et al., 2003).

A subpopulation of AFDSC discussed previously is CD117+, c-kit+, AF stem cells first isolated (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012). Cytocellular analysis shows that these cells are heavily marked with mesenchymal markers, but can differentiate into cells of all three germ layers (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012). CD117+ expression appears early in AFDSC from around 7 weeks of gestation with a peak expression at around 20 weeks gestation (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012). AF-derived stem cells are discussed in the discussion section below.

3.4.2.1 OCT-4 Pseudogene Expression In AFDSC

A study conducted by Ryan et al., mentioned above in the post-20th century historical perspectives on AF section, investigated the OCT-4 gene and its expression (Ryan, Pettit, Guillot, Chan, & Fisk, 2013). The Oct-4 gene has 3 variants: Oct-4A – variant 1; Oct-4 variants B and C, variants 2 and 3, respectively; are smaller variants found in cell cytoplasms with no known function (Ryan, Pettit, Guillot, Chan, & Fisk, 2013). Also, there are 6 pseudogenes with homology very close to Oct-4A, including the variants 2 and 3 above (Ryan, Pettit, Guillot, Chan, & Fisk, 2013). One of the pseudogenes transcriptionally active
within cells is Oct-4B (Ryan, Pettit, Guillot, Chan, & Fisk, 2013). OCT-4B's expression has been confirmed in cancer cells and adult bone marrow mesenchymal stem cells and term umbilical vein stem cells (Ryan, Pettit, Guillot, Chan, & Fisk, 2013). It is Oct-4A that expressed in embryonic carcinoma cells and embryonic cell lines, and thus gives “stemness” to these cells (Ryan, Pettit, Guillot, Chan, & Fisk, 2013). Studies wishing to identify the expression of Oct-4 use antibodies that do not discriminate between the Oct-4A gene and its pseudogene Oct-4B, which both show nuclear localization and have similar molecular weights (Ryan, Pettit, Guillot, Chan, & Fisk, 2013). Also, this study found conflicting reports in the literature on other stem cell markers similar to Oct-4 expression (Ryan, Pettit, Guillot, Chan, & Fisk, 2013). One of the biggest issues this study found was the lack of symmetry between mRNA expression and protein expression of stem cell markers including Sox-2, Rex-1, NANOG, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 (Ryan, Pettit, Guillot, Chan, & Fisk, 2013).
CHAPTER 4

DISCUSSION

The AM and AF possess complex and unique biological properties. These properties allow for the development and sustainability of the embryo and fetus. Most importantly, the AM and AF possess stem cells that have been utilized for diverse applications with far-reaching abilities and future potentials (Cananzi, Atala, & de Coppi, Stem cells from amniotic fluid, 2011; Da Sacco, De Filippo, & Perin, 2011; Dobreva, Pereira, Deprest, & Zwijsen, 2010). The unique qualities of these stem cells have allowed them and currently allow them to be utilized in regenerative medicine, tissue engineering, and gene therapy (Cananzi, Atala, & de Coppi, Stem cells from amniotic fluid, 2011; Da Sacco, De Filippo, & Perin, 2011; Dobreva, Pereira, Deprest, & Zwijsen, 2010). Research into AF-derived stem cells has been extensive, more so than that of amniotic AM stem cells. Although many properties of AF-derived stem cells are currently known their origin and reason for existence is not (Fauza, 2004).

A heterogeneous population of stem cells in AF is a hotly discussed topic among scholars. Evidence supporting such a population occurred in 2007 when de Coppi isolated and characterized CD117+ cells expressing Oct-4 with pluripotent properties from AF samples (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012). De Coppi emphasized that such cells were different from previously AMSC, fibroblast-like cells that expressed CD34 and CD105, markers
that were negative on CD117+ cells but had pluripotent and clonogeneic in vitro similarities (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012; Tsai, Lee, Chang, & Hwang, 2004). Such heterogeneity can be the result of stochastic phenotype switching in progeny of a clonal population, a phenomenon that is possibly occurring among AF stem cells (Salathe, Van Cleve, & Feldman, 2009).

Stochastic phenotype switching is a phenomenon responsible for phenotypic heterogeneity in a genetically uniform population of cells exposed to identical external environments (Salathe, Van Cleve, & Feldman, 2009; Raser & O'Shea, 2005). Such a phenomenon is believed to be an evolutionary adaptation that occurs independently of random mutations and recombination at the genetic level (Salathe, Van Cleve, & Feldman, 2009). This stochastic phenotype phenomenon has shown to play a very important and relevant role in drug tolerance phenotypes in bacterial populations, somatic evolution of cancer cells, and cell differentiation in embryonic stem cells (Rocco, Kierzek, & McFadden, 2013). Stochastic variation of cell populations, also defined as intrinsic molecular noise, is thought to be the result of the inherent random nature of biochemical reactions, specifically, gene expression and gene regulatory processes (Rocco, Kierzek, & McFadden, 2013; Raser & O'Shea, 2005). Ultimately, such stochastic fluctuations in gene expression and regulation heavily influence and determine downstream protein abundance (Raser & O'Shea, 2005). This variation in protein abundance within genetically identical cells of a population is thus responsible for phenotypic heterogeneity (Raser & O'Shea, 2005).
It is believed that stochastic phenotype switching is evolutionarily advantageous, thought to optimize the fitness of a genetically identical population in a constant fluctuating environment for which random mutation and other natural selection processes are unable to correctly prepare a population for unforeseen environmental changes (Acar, Mettetal, & van Oudenaarden, 2008). Mathematical technique and analyses have been used to make sense of stochastic phenotype switching (Rocco, Kierzek, & McFadden, 2013; Acar, Mettetal, & van Oudenaarden, 2008). Interestingly, cell populations with high stochastic phenotype switch rates outgrow cell populations with slow stochastic switch rates in highly fluctuating environments; however, cell populations with high stochastic phenotype switch rates are outgrown by cell populations with slow stochastic switch rates in low fluctuating or static environments (Acar, Mettetal, & van Oudenaarden, 2008). It is then assumed that stochastic phenotype switching within a specific identical cell population is heavily influenced by environmental change frequency (Acar, Mettetal, & van Oudenaarden, 2008). Also, original cell populations with slow transcriptional activity accounting for increased stochastic fluctuation but fast translational activity shows stable cell heterogeneity but cell populations with fast translational activity show a single phenotype distribution within the cell population (Rocco, Kierzek, & McFadden, 2013). Such a phenomenon can possibly explain the heterogeneity of stem cell phenotypes in amniotic fluid (Tsai, et al., 2007; Roubelakis, et al., 2007). The nature of amniotic fluid in the second semester,
which shows a wide variation in stem cell phenotype, is highly dynamic in fluid, molecule, and cell type, correlating such an event with the heterogeneous phenotype of stem cells in second trimester amniotic fluid (Roubelakis, et al., 2007; Modena & Fieni, 2004). The possibility of stochastic phenotype switching being the reason for the heterogeneity in amniotic stem cells one can then assume the origin of such cells is that of a single origin, which is still unknown. However, although phenotypes are heterogeneous, the morphology of such cells is consistent with the fibro-blast morphology identified by Kaviani et al. in 2001 (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012; Kaviani, Perry, Dzakovic, Jennings, Ziegler, & Fauza, 2001). It also assumes that heterogeneity phenotypes will differ with each normal AF sample, which is consistent with studies (Cananzi & De Coppi, CD117+ amniotic fluid cells., 2012; Roubelakis, et al., 2007).

It is also important to note that cells cultured previously in the 1970s and 1980s include stem cells that were unknown at the time (Dobreva, Pereira, Deprest, & Zwijsen, 2010; Gosden C. M., Amniotic fluid cell types and culture., 1983). A comparison of morphology and cytocellular phenotypes of the original classes of cells of AF cell culture to AFDSC could help elucidate the heterogeneity or homogeneity of AFDSC within AF (Dobreva, Pereira, Deprest, & Zwijsen, 2010; Gosden C. M., Amniotic fluid cell types and culture., 1983).
Discovering the origin of AFDSC would potentially put to rest whether the phenotypic heterogeneity of stem cells in AF is truly of a heterogeneous or homogenous clonal population. Cell markers greatly differ among AME cells, AM-ESC, and AFDSC (Tsai, et al., 2007). Although these cells are phenotypically different, it does not justify that such cells are not related nor are of the same origin. A more detailed analysis of the ontogeny of AF stem cells is required but should not rule out the AM as a possible source without sufficient evidence.

Stem cells most closely resembling AF stem cells, particularly AMSC, are of Wharton’s Jelly (WJ) of the umbilical cord (Jeschke, Gauglitz, Phan, Herndon, & Kita, 2011). WJ stem cells morphologically have a fibro-blast like appearance, similar to AMSC isolated by Kaviani in 2001 (Jeschke, Gauglitz, Phan, Herndon, & Kita, 2011; Kaviani, Perry, Dzakovic, Jennings, Ziegler, & Fauza, 2001). These cells also express cell surface markers including CD34 and CD105; and have pluripotent properties in which they are able to differentiate into all three germ layers (Jeschke, Gauglitz, Phan, Herndon, & Kita, 2011).
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Boston Children’s Hospital
• Dr. Dario Fauza’s lab is a surgical research lab that integrates the principles of both tissue engineering and stem cells, particularly, amniotic mesenchymal stem cells (aMSC), in the treatment of various congenital anomalies and medical pathologies
• Assisted surgical residents in various surgical procedures to produce effective experimental models and the treatment of induced models including instrument handling, scaffold and tissue placement, and fascia and dermal suturing
• Handled and cared for small and large animal species: rat, leporine, ovine, and porcine
• Animal anesthetic induction and pain medication administration
• Routes of administration include subcutaneous and intraperitoneal
• Gavage administration to rodent models
• Independent research project and proposal in limb regeneration utilizing amniotic mesenchymal stem cells; hypothesis and supporting literature can be found at http://www.gofundme.com/2rp9aq
• Independent research in scar-less wound healing, and amniotic mesenchymal stem cell origin identification using cellular phylogenetic algorithms for cell lineage reconstruction.
Bacteriologist I/Microbiologist
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Massachusetts Department of Public Health
- Performed a variety of highly complex microbiological tests to identify and characterize the presence of biological agents and other infectious diseases, including DNA extraction and real-time polymerase chain reaction (qPCR)
- Assured that incoming specimens were recorded, processed, and triaged appropriately while maintaining "chain-of custody"
- Maintained documentation collection on findings and protocols
- Interpreted and reported test results to appropriate individuals using established communication protocols with Local, State, and Federal agencies
- Performed quality control and quality assurance procedures such as routine equipment maintenance and calibration, documentation of quality control and quality assurance activities
- Provided laboratory support for field responses and investigations as directed
- Maintained proficiency in aseptic methods and molecular diagnostics methods in both bio-safety level 2 and bio-safety level 3 environments
- Provided laboratory support for field responses and investigations as assigned by supervisor
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Laboratory Assistant
March 2005 - December 2009
Brigham & Women’s Hospital, Advanced Center for Molecular Diagnostics
- Performed RT-PCR on patient samples to generate templates for sequence analysis
- DNA and RNA isolation and extraction
- Spectrophotometer utilization to measure DNA and RNA content
- Performed quality control for PCR instruments
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- Performed routine maintenance on laboratory equipment such as bench top instruments and fume hoods
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Awards
Professional development award (2013) – Outstanding community service
Dean’s list (2007, 2008)

Textbook Chapter
Historical perspective chapter of amniotic membrane stem cells and amniotic fluid stem cells in preparation for amniotic stem cell textbook. Chief editor is Dario O. Fauza MD, PhD of Boston Children’s Hospital. Springer Publishing Company.

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Manuscript complete, under journal review:

- “Dynamic Alterations in Hippo Signaling Pathway and YAP Activation during Liver Regeneration”. James Grijalva, Megan Huizenga, Kaly Mueller, Steven Rodriguez, Joseph Brazzo, Fernando Camargo, Ghazaleh Sadri-Vakili, and Khashayar Vakili has been sent to the Editor-in-Chief for assignment.

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- “Partial or Complete Coverage of Experimental Spina Bifida by Simple Intra-Amniotic Injection of Concentrated Amniotic Mesenchymal Stem Cells”. Beatrice Dionigi, MD; Azra Ahmed, BS; Joseph Brazzo III, BS; John Patrick Connors, BS; David Zurakowski, PhD; Dario O Fauza, MD, PhD

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