ROLE OF EXOSOMAL ANNEXIN A2 IN ANGIOGENESIS AND BREAST CANCER METASTASIS

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DISSERTATION

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LIST OF ABBREVIATIONS

APL – acute promyelocytic leukemia

AIIt – AnxA2 heterotetramer

AnxA2 – Annexin A2

BLI – bioluminescence imaging

DMEM – Dulbecco's Modified Eagle Medium

ε- ACA – ε-amino caproic acid

ELISA - Enzyme-Linked Immunosorbent Assay

Exo-AnxA2 – Exosomal AnxA2

ECM – extracellular matrix

EGF – Epidermal Frowth Factor

HSP70 - Heat Shock Protein 70

HSC70 - Heat Shock Cognate 70

H & E – Hematoxylin and Eosin

ic – intracardiac

IP – Intraperitoneal injection

MVB – Multivesicular bodies

MMP 9– matrix metalloproteinase 9

NFκB – Nuclear factor κB

phospho-NFκB – phospho Nuclear factor κB

(PtdIns(4,5)P2) – phosphatidylinositol-4,5 bisphosphate

PAI1 – plasminogen activator inhibitor-1

PBS – Phosphate buffered saline

PAI-2 – plasminogen activator inhibitor-2

phospho-STAT3 – phospho Signal transducer and activator of transcription 3

p38 – p38 mitogen-activated protein kinases

phospho-p38 – phospho p38 mitogen-activated protein kinases

STAT3 – Signal transducer and activator of transcription 3

tPA – tissue-type plasminogen activator

TN-C – tenascin C

uPA – urokinase plasminogen activator

VEGF – Vascular Endothelial Growth Factor

VEGFR1 – Vascular Endothelial Growth Factor Receptor 1

WT- Wild Type

CHAPTER I. INTRODUCTION

A. **1) Membrane vesicles:** Membrane vesicles are spherical structures that are limited by a lipid bilayer. In eukaryotic cells, carrier vesicles can transfer components between intracellular compartments by budding from the membrane of a donor compartment, traveling in the cytoplasm before fusing with the membrane of an acceptor compartment. These carrier vesicles contain material from the lumen of the donor compartment and expose the cytoplasmic side of this compartment at their outer surface. All these vesicles remain strictly intracellular. On the other hand, cells can generate membrane vesicles that are secreted into the extracellular space; such vesicles can form either at the plasma membrane or in the lumen of internal compartments. These vesicles contain cytosol and their membrane orientation is the same as that of the donor cell, hence, they can be considered to be miniature versions of a cell. There are various types of secreted membrane vesicles that have distinct structural and biochemical properties depending on their intracellular site of origin, and these features probably affect their function (Thery et al. 2009). TABLE 1 provides an overview of the main characteristics of each type of vesicle that is released by live cells and Fig. 1 depicts the origin of the most commonly found vesicles.

Feature*	Exosomes	Microvesicles	Ectosomes	Membrane particles	Exosome-like vesicles	Apoptotic vesicles
Size	50–100 nm	100–1,000 nm	50–200 nm	50–80 nm	20–50 nm	50–500 nm
Density in sucrose	1.13–1.19 g/ml	ND	ND	1.04–1.07 g/ml	1.1 g/ml	1.16–1.28 g/ml
Appearance by electron microscopy‡	Cup shape	Irregular shape and electron-dense	Bilamellar round structures	Round	Irregular shape	Heterogeneous
Sedimentation	100,000 g	10,000 g	160,000–200,000 g	100,000–200,000 g	175,000 g	1,200g , 10,000 g or 100,000 g
Lipid composition	Enriched in cholesterol, sphingomyelin and ceramide; contain lipid rafts; expose phosphatidylserine	Expose phosphatidylserine	Enriched in cholesterol and diacylglycerol; expose phosphatidylserine	ND	Do not contain lipid rafts	ND
Main protein markers	Tetraspanins (CD63, CD9), Alix and TSG101	Integrins, selectins and CD40 ligand	CR1 and proteolytic enzymes; no CD63	CD133; no CD63	TNFRI	Histones
Intracellular origin	Internal compartments (endosomes)	Plasma membrane	Plasma membrane	Plasma membrane	Internal compartments?	ND
Main reference	142	3	143	8	17	144

*The listed features of vesicles secreted by live cells are based on observation of preparations of 100% pure vesicles. However, in practice, all vesicle preparations are heterogeneous, with different protocols allowing the enrichment of one type over another, and they can be classified according to the presence of several (but not necessarily all) of the listed features. ‡Appearance by electron microscopy is only an indication of vesicle type and should not be used to define vesicles, as their microscopic appearance can be influenced by the fixation and phase contrast techniques used. CR1, complement component receptor 1; ND, not determined; TNFRI, tumour necrosis factor receptor I; TSG101, tumour susceptibility gene 101.

Thery et al. 2009, Nature Reviews Immunology

Table 1 **Table 1: List of most commonly found membrane vesicles and their characteristics.**

A. **2)** Exosomes: Secreted vesicles known as exosomes were first discovered nearly 35 years ago. But, considered little more than cellular garbage cans acting to discard unwanted molecular components, exosomes remained little studied for the next 10 years. However, research in the past few years has highlighted that the vesicles are like signaling payloads containing cell-specific collections of proteins, lipids, and genetic material that are transported to other cells where they alter function and physiology, possibly through – direct stimulation of target cells via surface-expressed growth factors or bioactive lipids, transfer of membrane receptors, or delivery of proteins or RNAs to target cells.

Exosomes represent a specific subtype of secreted membrane vesicles and there are at least 6 different types of membrane vesicles (Stoorvogel et al. 2002, Thery et al. 2002). Exosomes are formed in endosomal compartments called multivesicular endosomes, containing internal vesicles that package and store molecules in membrane-bound structures.

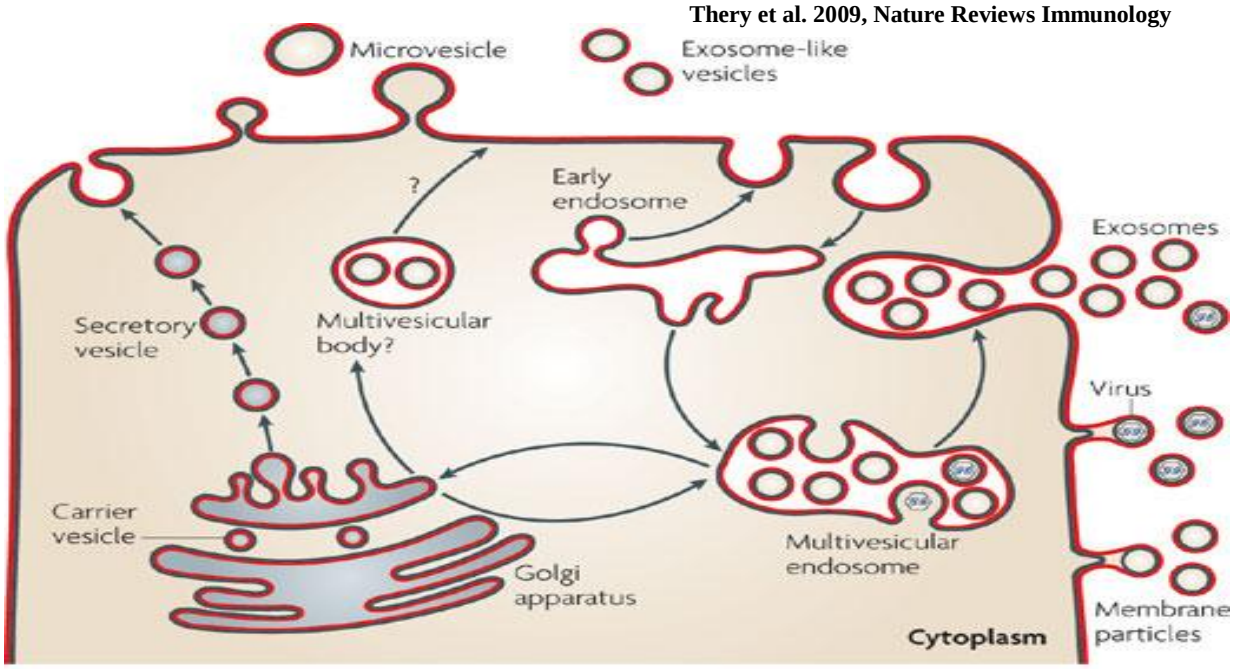


Fig.1: Cartoon depicting the origin of most commonly found membrane vesicles.

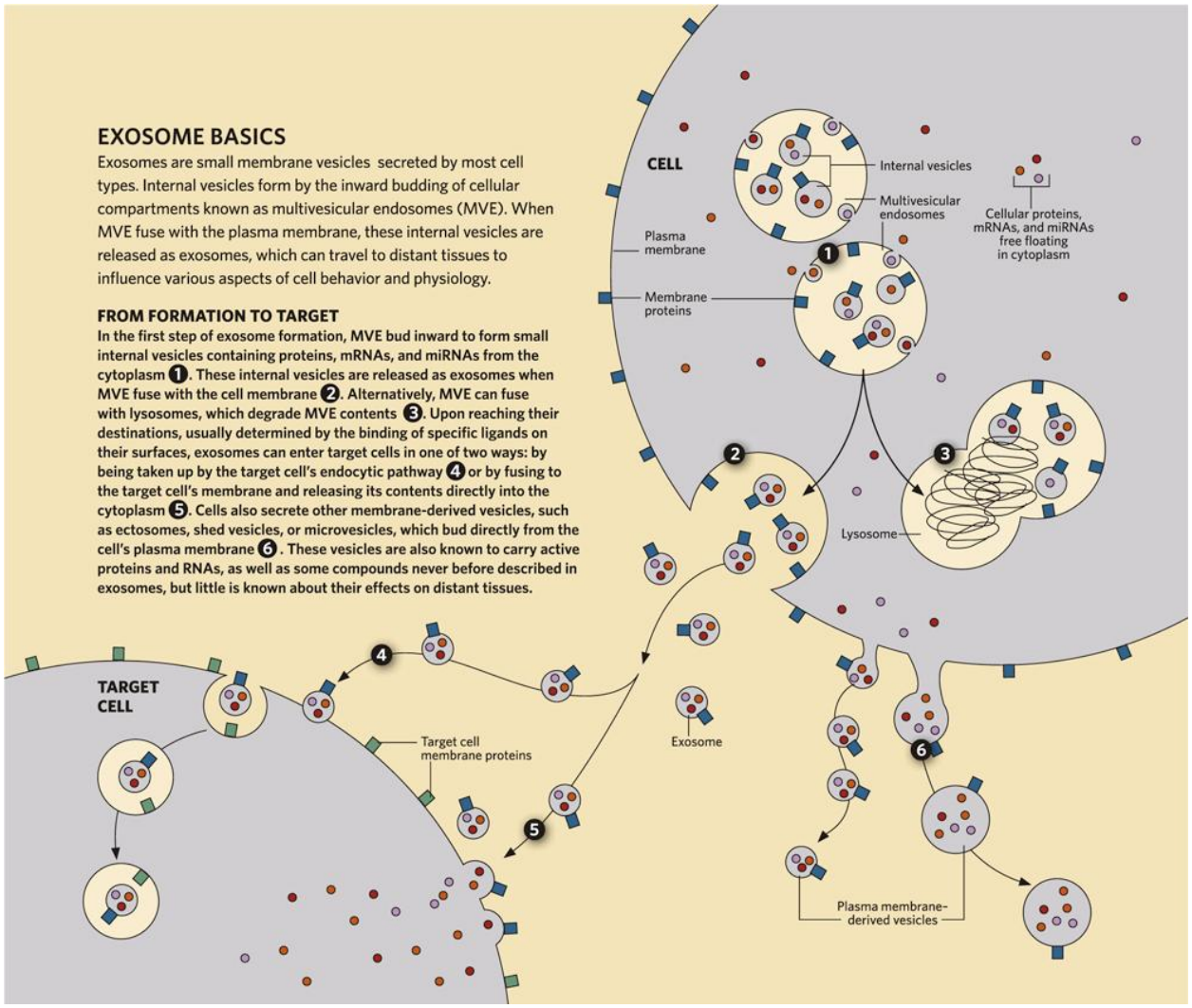


Fig.2: Exosome Basics **Thery 2011, F1000 Biol. Reports**

A. **3) Exosomes and cancer:** Recent studies with exosomes have highlighted their role in various diseases, especially cancer. Tumor-derived exosomes have been viewed as a source of tumor antigens that can be used to induce antitumor immune responses (Andre et al. 2002, Dai et al. 2005, Wolfers et al. 2001). However, tumor-derived exosomes also have been found to possess immunosuppressive properties and are able to facilitate tumor growth, metastasis, and the development of drug resistance. These different effects of tumor-derived exosomes contribute to the pathogenesis of cancer. By stimulating angiogenesis (Grange et al. 2011, Hegmans et al. 2004, Peinado et al. 2012, Wysoczynski et al. 2009), modulating stromal cells

(Castellana et al. 2009), and remodeling extracellular matrix (Graves et al. 2004, Dolo et al. 1999), tumor-derived exosomes have been found to contribute to the establishment of a premetastatic niche, generating a suitable microenvironment in distant metastatic sites. (Peinado et al. 2012, Peinado et al. 2011) Gynecologic neoplasias, including ovarian cancer and breast cancer exosomes were found to contain metalloproteinases that have proteolytic activity. Thus exosomes can increase extracellular matrix degradation and augment tumor invasion into the stroma (Nieuwland et al. 2010, Runz et al. 2007). Interestingly, the number of exosomes secreted by tumor cells has been found to be more than normal cells. (Kharaziha et al. 2012)

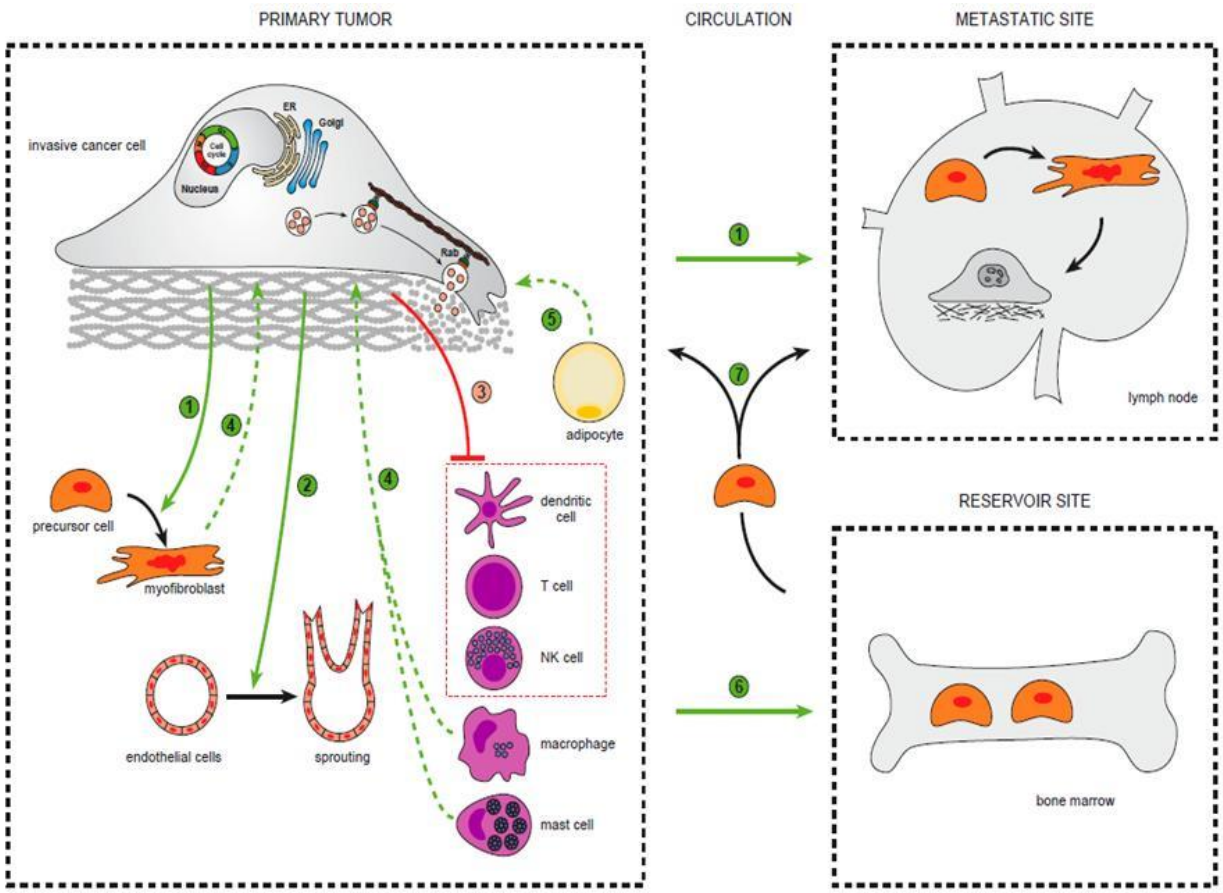


Fig.3: Effects of tumor and stromal exosomes Hendrix A, Hume AN. Exosome signaling in mammary gland development and cancer. *Int J Dev Biol*. 2011;55(7-9):879-887.

A. 4) Mechanisms by which the cancer cell and stromal cell-derived exosomes can affect the primary tumor and the distant reservoir and metastatic sites: Cancer-cell derived exosomes trigger myofibroblast differentiation (1), endothelial sprouting (2) and immune suppression (3) in the primary tumor. Cancer cell derived exosomes enter the circulation and home to metastatic sites (e.g. lymph node) to optimize conditions for cancer cell deposition and growth (1). Stromal cell-derived exosomes stimulate invasive growth (4) and cellular metabolism (5). Exosomes secreted by the primary tumor contribute to bone marrow cell education and mobilization (6) and recruit bone marrow progenitor cells to the primary tumor and metastatic sites (7) (Fig. 3).

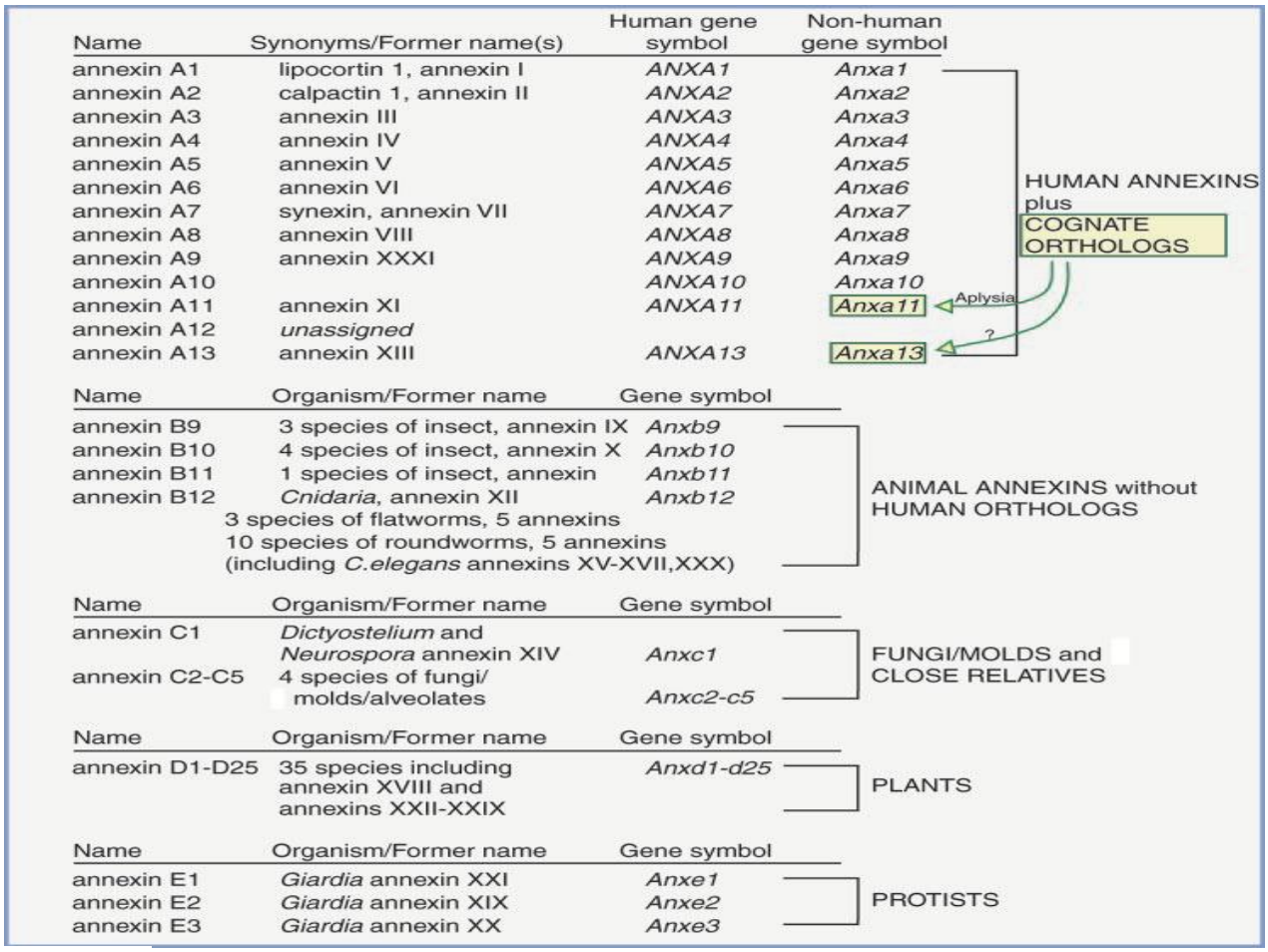
A. 5) Exosomal Proteins as Biomarkers: Exosomes are enriched in a selective repertoire of proteins and nucleic acids from parental cells and are thought to be actively involved in conferring intercellular signals. Almost 4,500 proteins have been detected in exosome fractions via proteomic profiling of exosomes from a variety of cell types (Simpson et al. 2012). But, very little is known regarding the functional relevance of these exosomal proteins, especially their role in modulating the cellular environment. With increasing number of studies indicating the importance of exosomes, there is an urgent need to characterize and study the function of the exosomal proteins under physiological and more importantly, under pathological conditions. Having a better understanding of the functional relevance of the exosomal proteins will serve two main purposes: firstly, it will help us understand how the cellular microenvironment works under both physiological and pathological conditions; secondly, this understanding can lead to identification of specific biomarkers which can be used for disease screening, prognosis and even targeted therapy. Cancer is one such disease, where the role of the surrounding tumor

microenvironment has been implicated as equally important in promoting tumorigenesis as the cancer itself (Klemm and Joyce, 2015)

B. Annexin and their structures:

B. 1) Annexins: A multigene family of Ca^{2+} dependent phospholipid binding proteins

Annexins comprise a multigene family of Ca^{2+} and phospholipid binding proteins with markedly conserved structural homology among species and divergent roles in many biological processes (Moss and Morgan 2004). The annexin family comprises of more than 500 different gene products and 400 widely conserved sequenced proteins (Morgan and Fernandez 1997) (Table 2). In vertebrates 12 annexins have been classified under the annexin A family termed as annexin A1-A13 (AnxA1-A13) leaving AnxA12 unassigned in the original nomenclature (Morgan and Fernandez 1997). The annexin family of proteins is known to be encoded by 1-20 annexin genes in most eukaryotic species (Moss and Morgan 2004). In humans there are 12 Annexin genes (AnxA1-A12), which are dispersed throughout the genome and range in size from 15 kb (AnxA9) to 96 kb (AnxA10) (Moss and Morgan 2004, Barton et al. 1991).



Name	Synonyms/Former name(s)	Human gene symbol	Non-human gene symbol
annexin A1	lipocortin 1, annexin I	*ANXA1*	*Anxa1*
annexin A2	calpactin 1, annexin II	*ANXA2*	*Anxa2*
annexin A3	annexin III	*ANXA3*	*Anxa3*
annexin A4	annexin IV	*ANXA4*	*Anxa4*
annexin A5	annexin V	*ANXA5*	*Anxa5*
annexin A6	annexin VI	*ANXA6*	*Anxa6*
annexin A7	synexin, annexin VII	*ANXA7*	*Anxa7*
annexin A8	annexin VIII	*ANXA8*	*Anxa8*
annexin A9	annexin XXXI	*ANXA9*	*Anxa9*
annexin A10		*ANXA10*	*Anxa10*
annexin A11	annexin XI	*ANXA11*	*Anxa11*
annexin A12	*unassigned*		
annexin A13	annexin XIII	*ANXA13*	*Anxa13*

HUMAN ANNEXINS plus COGNATE ORTHOLOGS

Aplysia

?

Name	Organism/Former name	Gene symbol
annexin B9	3 species of insect, annexin IX	*Anxb9*
annexin B10	4 species of insect, annexin X	*Anxb10*
annexin B11	1 species of insect, annexin	*Anxb11*
annexin B12	*Cnidaria*, annexin XII	*Anxb12*
	3 species of flatworms, 5 annexins	
	10 species of roundworms, 5 annexins (including *C.elegans* annexins XV-XVII,XXX)	

ANIMAL ANNEXINS without HUMAN ORTHOLOGS

Name	Organism/Former name	Gene symbol
annexin C1	*Dictyostelium* and *Neurospora* annexin XIV	*Anxc1*
annexin C2-C5	4 species of fungi/ molds/alveolates	*Anxc2-c5*

FUNGI/MOLDS and CLOSE RELATIVES

Name	Organism/Former name	Gene symbol
annexin D1-D25	35 species including annexin XVIII and annexins XXII-XXIX	*Anxd1-d25*

PLANTS

Name	Organism/Former name	Gene symbol
annexin E1	*Giardia* annexin XXI	*Anxe1*
annexin E2	*Giardia* annexin XIX	*Anxe2*
annexin E3	*Giardia* annexin XX	*Anxe3*

PROTISTS

Table. 2 Volker Gerke and Stephen E. Moss, *Physiol Rev*, 2002

Table 2. The new annexin nomenclature. The five major annexin groups (A–E) are shown, with details of the most extensively studied family members. The nomenclature is that proposed by Reg Morgan and Pilar Fernandez and endorsed by participants at the 50th Harden Conference on Annexins held at Wye College, UK, September 1–5, 1999.

B. 2) Molecular structure of annexins:

In response to several stimuli, annexins have the ability to bind to the cellular membranes in a reversible manner owing to their unique structure (Rescher and Gerke 2008). All annexins are characterized by a bipartite structure with a variable amino terminal domain and a conserved carboxyl terminal domain (Gerke and Moss 2002) (Fig. 4). The Ca^{2+} and phospholipid-binding activities of the annexins are based on their C-terminus whereas the amino terminus is the site for extensive post translational modifications and interactions with other proteins (Gerke and Moss 2002). Except for AnxA6, all annexins posses four repeats of approximately 70 amino

acids in their carboxyl terminus. Each of these repeats possess an α-helical domain that comprises a Ca^{2+}-binding site (Gerke et al. 2005). The ability to interact with the membranes in a regulated manner in response to changes in intracellular Ca^{2+} concentrations with varying affinities is dependent on these sites (Burgoyne and Geisow, 1989). Binding to Ca^{2+} causes a conformational change of the α-helical domain forming a compact, slightly curved disc that possesses a hydrophobic convex surface and a concave surface. The convex surface harbors the Ca^{2+} and membrane binding sites whereas the concave surface points away from the membrane and is available for interaction with other proteins (Rosengarth and Luecke, 2003). However the exact molecular events that contribute to these changes in the structure of annexins is still unclear.

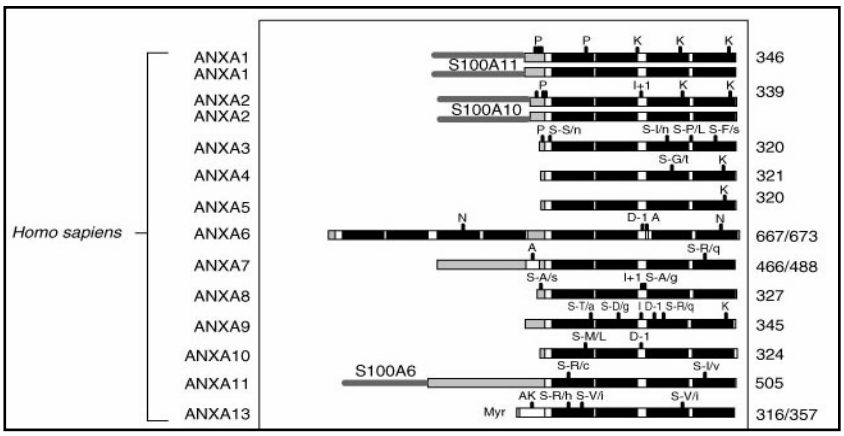


Fig. 4 Domain structure of the annexin family of proteins Genome Biology, 2004

Figure 4. Domain structure of the annexin family of proteins: The bipartite structure of the 12 human annexins showing the four conserved Ca^{2+}-binding repeats (black). The variable N- terminal domain (shaded) which differs in length and sequence. Interaction of AnxA1, AnxA2 and AnxA11 with the S100 family of proteins (S100A11, S100A10 and S100A6 respectively). P – phosphorylation sites; K – KGD synapomorphy (a

conserved, inherited characteristic of proteins); I – codon insertions (+x denotes the number of codons inserted); S-A/b, nonsynonymous coding polymorphisms (SNPs) with the amino acid in the major variant (A) and that in the minor variant (b); N – putative nucleotide-binding sites; D – codon deletions (-x denotes the number of codons deleted); A – alternatively spliced exons; Myr – myristoylation. The total amino acid length of each protein is indicated on the right. (Moss et al. 2004)

B. 3) Characteristics of the variable N-terminal domain of annexins

The N-terminal domain of annexins is diverse in sequence and length among the members of the annexin family. Binding of the annexin proteins to the membrane leads to the folding of the N-terminus into a structurally separate unit on the concave side of the membrane and opposite to the membrane-binding assembly (Swairjo and Seaton 1994). This site is not only responsible for regulating the interactions with other protein ligands but is also important for the AnxA2 membrane assembly in response to different intracellular stimuli (Raynal and Pollard 1994). For example, the N-terminus of AnxA2 regulates its binding to the EF-hand superfamily of Ca^{2+}-binding proteins (Gerke and Moss 2002, Gerke et al. 2005). AnxA1 and AnxA2 also interact with the S100 family members like S100A11 and S100A10 respectively which are characterized by two consecutive EF hand domains (Heizmann 2002). The N-terminal region of annexins are also responsible for forming a binding pocket for its partners as seen in case of AnxA2, where it forms a binding pocket for the binding of the S100 dimer subunits (Rety et al. 1999). The resulting structure is a highly symmetrical and stable molecular entity comprising of two central S100 dimers connecting two annexin monomers (Rety et al. 1999). Such a molecular structure is assumed to provide stability to the annexin membrane complexes by bridging two membrane bound core

domains by a S100 dimer (Lewit-Bentley, Rety 2000). Apart from contributing to the stability of the annexin membrane complexes the N-terminus is also a site for several protein-protein interactions and post- translational modifications (Gerke and Moss 2002). The different types of post translational modifications reported for the annexin family include miristoylation of AnxA13 and serine/threonine as well as tyrosine phosphorylation of several annexins including annexins A1, A2, A4, A6 and A7 (Gerke and Moss 2002, Gerke et al. 2005).

C. Annexin family of membrane-bound proteins with diverse functions

C. 1) Annexins as membrane scaffolding proteins

One important aspect of the unique structures of the annexin family of proteins is that it enables them to bind to the acidic phospholipids in a Ca^{2+}-dependent manner (Raynal and Pollard 1994). Owing to the unique property of the N-terminus, many members of the annexin family can bind to the cellular membranes either as single molecules or as protein complexes (Gerke et al. 2005). AnxA1 and AnxA2 have been specifically found to bind to the phospholipid bilayers rich in phosphatidyl serine/phosphatidyl choline mixtures and form monolayer protein clusters that are amorphous and mobile across the bilayers (Lambert et al. 2004). Interestingly, binding of annexins to the negatively charged phospholipids has been shown to be followed by segregation of membrane lipids and recruitment of actin assembly within the annexin clusters underneath the plasma membrane (Hayes et al. 2009). AnxA2 has been shown to preferentially bind to phosphatidylinositol-4,5 bisphosphate (PtdIns(4,5)P2) and this binding leads to the formation and stabilization of actin assembly sites at cellular membranes (Hayes et al. 2009). Although there has been a lot of studies on the mechanism of AnxA2 binding to the membrane, it is still unclear whether AnxA2 preferentially localizes to

the (PtdIns(4,5)P2) rich regions of the membrane or its binding leads to the segregation and formation of negatively charged phospholipid clusters.

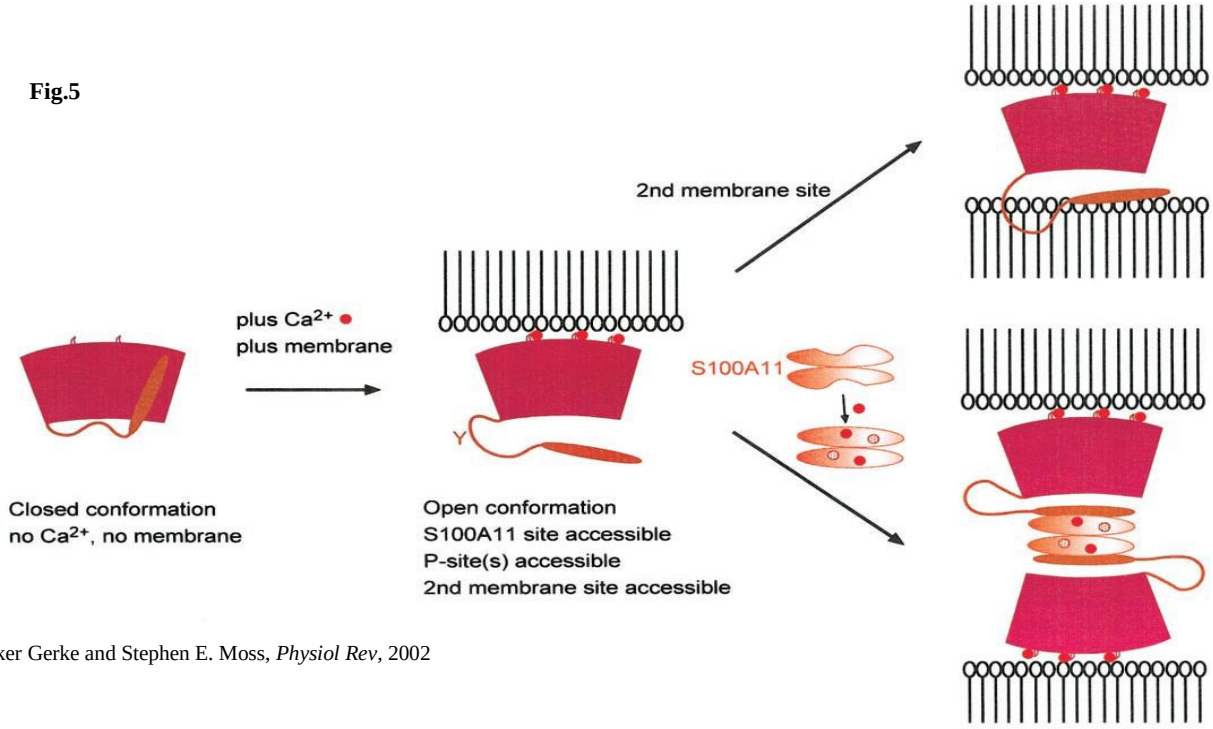
Another important characteristic of most of the annexin family of proteins is that they can aggregate the vesicular and plasma membrane in a Ca^{2+}-dependent manner (Gerke et al. 2005) most probably as a result of the interactions between the core domains of annexins on both the membranes (Gerke and Moss 2002). For example, the S100 proteins of the heteromeric complex acts as a bridge to bring together the two core domains in case of annexin family members AnxA2 and AnxA7 which form a heteromeric complex with the S100 family of proteins S100A10 and S100A11 respectively (Rety et al. 1999, Lewit-Bentley, Rety 2000).

C. 2) Annexin membrane interactions

Although annexins are freely soluble proteins distributed in the cytosol of resting cells under physiological Ca^{2+} concentrations, in response to elevated levels of intracellular Ca^{2+} it has been shown that they localize to the membranes (Burgoyne and Geisow, 1989). In response to specific stimuli that induce mobilization of intracellular Ca^{2+} annexin family of proteins have been reported to localize to cellular membranes, although there is a lot of variability among the different annexin members regarding the concentrations of free Ca^{2+} required for the membrane translocation (Barwise et al. 1996). Thus the recruitment of annexins to their target membranes is dependent upon the levels of intracellular Ca^{2+}, its location as well as the mode of Ca^{2+} mobilization and finally the signaling events that induce the levels of intracellular Ca^{2+} (Barwise et al. 1996, Blanchard et al. 1996). The cells can respond to divergent stimuli that increase the levels of intracellular Ca^{2+} concentrations by undertaking a range of highly dynamic membrane reorganizations supported by AnxA2-

induced membrane scaffolds (Burgoyne and Geisow, 1989). Although a majority of the annexin family of proteins interact with cellular membranes in a Ca^{2+}-dependent manner, there are many exceptions to this expected pattern. It has been shown that many other factors such as heat stress and changes in cellular pH also influence the membrane-binding ability of annexins (Raynal and Pollard 1994). Furthermore, the presence of the C- terminal Ca^{2+}-binding sites is essential for the binding of annexins to cellular membranes in a Ca^{2+}-dependent manner and some annexins such as AnxA9 and AnxA10 are not affected by intracellular Ca^{2+} concentrations as they do not possess the C-terminal Ca^{2+}-binding sites (Gerke et al. 2005, Goebeler et al. 2003) (Fig. 5).

Fig.5



Volker Gerke and Stephen E. Moss, *Physiol Rev*, 2002

Figure 5. Model describing the switch of helix D in the annexin A1 structure and its implications for membrane aggregation. In the crystal structure of Ca2+ free annexin A1 (red), the NH2-terminal α-helix, which contains the S100A11binding site (brown), is replacing helix D of the third repeat. Ca2+ dependent membrane binding could be accompanied by a conformational change establishing the Ca2+ bound crystal structure of the annexin A1 core and, most likely, a more accessible NH2-terminal domain. As a result, the NH2-terminal domain can interact with a second membrane surface or the S100A11 dimer, which itself requires Ca2+ binding to establish an interaction-competent conformation.

C. 3) Binding of annexins to endosomal membranes

It has been reported that AnxA1, AnxA2 and AnxA6 have unique endosomal targeting sequences at their N-terminus that enables them to interact with the endosomal membranes (Emans et al. 1993). Although most of the annexin members bind to the membrane in a Ca^{2+}-dependent manner, some members particularly AnxA2 has been shown to bind to the endosomal membranes in the presence of Ca^{2+} chelator agents (Jost et al. 1997). More importantly, this Ca^{2+}-independent binding of AnxA2 to the endosomal membrane does not require the N-terminus binding of p11 and is also independent of the Ca^{2+}-binding sites in the C-terminus of AnxA2 (Emans et al. 1993). However, it is dependent on the phosphorylation of certain N-terminal tyrosine residues of AnxA2 (Morel and Gruenberg 2009). This Ca^{2+}-independent atypical binding of AnxA2 has been also reported to be seen in regions of the endosomal membranes rich in cholesterol and sphingolipids (Zeuschner et al. 2001).

C. 4) Extracellular annexin activities:

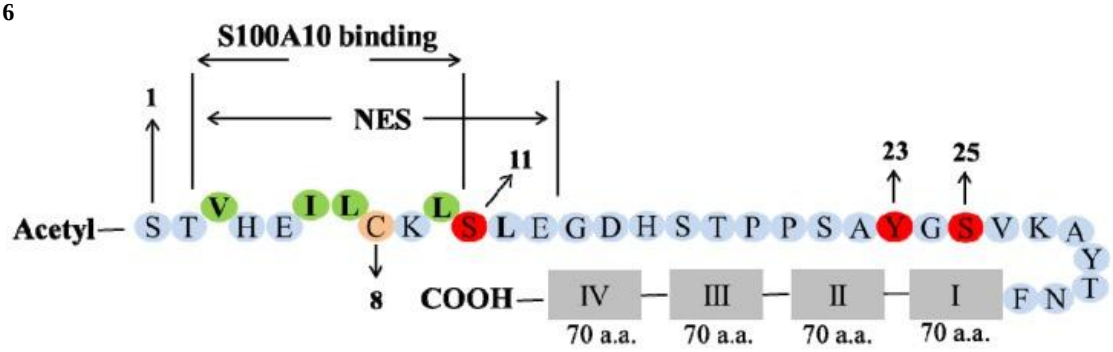
Annexins are soluble cytosolic proteins which lack signal sequences for directing them into the classical secretory pathway. Nevertheless, some annexin members have been reported to be present in extracellular fluids. Alternative pathways for the secretion of annexins A1 and A2 have been proposed (Castro-Caldas et al., 2002; Chapman et al. 2003; Danielsen et al. 2003; Faure et al., 2002; Zhao et al., 2003). Interestingly, several binding sites for extracellular annexins have been discovered on the cell surface and several possible extracellular functions for these proteins have been proposed; including the role of AnxA5 as an anti-coagulant protein, role of AnxA2 as an endothelial cell-surface receptor for plasminogen and tissue-type plasminogen activator (tPA), as well as an anti-inflammatory role for AnxA1. AnxA5 has been reported to bind to phosphatidylserine exposed on the surface of syncytiotrophoblasts. As mentioned earlier,

AnxA2 can act as a receptor for plasminogen and tPA on the surface of endothelial cells and leukocytes and thereby act as a positive modulator in the fibrinolytic cascade (Kim and Hajjar, 2002).

D. Structure of AnxA2:

Similar to other annexin members, AnxA2 is also composed of two major domains, the amino-terminal domain or head region which is highly variant and the conserved carboxyl-terminal core domain. The amino-terminal region is responsible for ligand and protein interactions as well as the site for post-translational modification, while carboxyl-terminal core region is responsible for binding of calcium and anionic phospholipids, DNA, F-actin and heparin (Bharadwaj et al. 2013) (Fig. 6). The carboxyl-terminal core domain consists of four repeat segments which are made up of 70 amino acids; each segment being called the annexin repeat. Each repeat segment in turn contains five α-helices (A–E). Four of these α-helices are oriented anti- parallel and the fifth α-helice is oriented perpendicular to them wound in a right-handed superhelix. The amino-terminal tail domain of AnxA2 contains a region of amphipathic alpha-helix which binds to S100A10 through its hydrophobic surface. Moreover *N*-acetylation of AnxA2 is also required for forming the complex with S100A10.

Fig. 6



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Val 3, Ile 6, Leu 7 and Leu 10 – S100A10 binding hydrophobic residues
Ser 11 – PKC phosphorylation site
Tyr 23 – pp60Src phosphorylation site
Ser 25 – PKC phosphorylation site
Cys 8 – Redox active cysteine
Carboxyl domain – calcium, phospholipid, membrane, F-actin and heparin binding site

Figure 6. Domain structure of annexin A2. Annexin A2 is composed to two domains—the amino-terminal domain and carboxyl-terminal domain. The amino-terminal is the site for post-translational modifications (Ser-1–Phe-32) such as acetylation (Ser-1) and phosphorylation (Ser-11, Tyr-23, Ser-25). Additionally it also encompasses the redox reactive cysteine residue (Cys-8) and the nuclear export sequence (NES) (Val-3-Leu-12). The S100A10 binding site is an amphipathic α-helix, with the hydrophobic residues, Val-3, Ile-6, Leu-7 and Leu-10 making contacts with S100A10. The carboxyl-terminal core domain includes four predominantly alpha-helical domains each containing 70 amino acids. This carboxyl-terminal core domain contains binding sites for heparin and RNA, calcium and phospholipid and as well as for F-actin.

E. Potential biological roles of extracellular AnxA2

E. 1) Plasminogen activation

AnxA2 binds to tissue plasminogen activator (tPA) and plasminogen by acting as a profibrinolytic receptor and facilitates in the generation of plasmin (Cesearman et al. 1994). According to the reported kinetic studies addition of soluble AnxA2 to tPA and plasminogen *in vitro*, increases the rate of plasmin generation by ~60 fold (Cesearman et al. 1994). Hajjar et al. has reported that AnxA2 knockout mice show a complete reduction in the

generation of extracellular plasmin on the surface of endothelial cells and show an incomplete reduction of injury-induced arterial thrombi (Ling et al. 2004) Furthermore, in leukemic cells derived from acute promyelocytic leukemia (APL) patients, overexpression of AnxA2 has been reported on the surface. This has been attributed to dysregulated fibrinolytic activity and it correlates with the clinical manifestations of bleeding (Menell et al. 1999). In addition, emerging evidence indicates that expression of AnxA2 on the surface of monocytes and macrophages contribute to the ability of the cells to degrade the extracellular matrix (ECM) and migrate to sites of inflammation (Brownstein et al. 2004). Binding studies have shown that the presence of lysine residues in plasminogen is essential for its binding to AnxA2 and it was abrogated by 90-95% in the presence of a lysine analog €-amino caproic acid (€- ACA) and also by the treatment of AnxA2 with carboxypeptidase B indicating that the binding occurs by a C-terminal lysine-dependent mechanism (Kim and Hajjar 2002). In contrast the binding of tPA to AnxA2 is €-ACA resistant and occurs via a high affinity binding site on tPA and binding to AnxA2 also confers protection on tPA from its physiological inhibitor, plasminogen activator inhibitor-1 (Herren et al. 2003).

E. 2) AnxA2 and its role in membrane organization and traffic

Apart from playing an important role in membrane stabilization, AnxA2 has also been reported to bind with F- actin localized to the actin rich regions of the membrane (Hayes et al. 2006). By virtue of its interaction with actin cytoskeleton, AnxA2 helps in propelling the endocytic vesicles form the plasma membrane to the cell interior (Morel et al. 2009). The interaction between AnxA2 and the actin-polymerizing platforms is reported to be dependent on Ca^{2+} and requiring a carboxyl terminal sequence located in the third repeat of AnxA2

(Rescher et al. 2009). This carboxyl terminal sequence is highly conserved among the annexin family of actin-binding proteins except for AnxA4 which does not bind F-actin (Rescher et al. 2009).

Interestingly, although AnxA2 binds to membrane actin filament bundles, it has not been found to be associated with the cytoplasmic actin bundles (Hayes et al. 2006). This suggests that the actin-binding activity of AnxA2 is restricted to the assembly of actin structures at the cellular membranes. It is reported that AnxA2 has to bind to the cellular membranes in order to be recruited to the actin–assembly platforms (Hayes et al. 2006). Structural studies have revealed that most actin-assembly structures beneath the plasma membrane share raft-like characteristics (Babiychuk and Draeger 2000). However it is unclear whether AnxA2 laterally diffuses to the membrane raft structures when there is an active actin–polymerization process or it is preferentially localized to the raft regions of the membrane (Rescher and Gerke 2004). Also biochemical studies have reported that AnxA2 can preferentially bind to the negatively charged membrane microdomains with similar affinity to that of many pleckstrin-homology domain proteins and with high specificity (Hayes et al. 2004). These indicate that AnxA2 might play an important role in the organization of raft-like membrane microdomains at sites of actin- assembly and this organization plays an important role in organization of membrane structure as well as cellular signaling (Rescher et al. 2004).

E. 3) AnxA2 and Tenascin C (TN-C)-mediated signal transduction

Tenascin C is an extracellular matrix protein with a spatially and temporally restricted tissue distribution implicated in guidance of migrating neurons as well as axons during development, synaptic plasticity, and neuronal regeneration. The large tenascin C (TN-C)

splice variant has been implicated in several biological functions including active cell migration and tissue remodeling (Esposito et al. 2006). Interestingly AnxA2 has been identified as a candidate receptor for the alternately spliced segment of TN-C (Chung and Erickson 1994). Interaction between AnxA2 and TN-C induces loss of focal adhesion, migration and induction of mitogenic response in endothelial cells (Chung and Murphy-Ullrich et al. 1996). There have been specific reports where AnxA2 and TN-C splice variants are found to be overexpressed in tumors and their expression is correlated with the increase in the migratory and invasive potential of cancer cells. This indicates a possible interaction between the two proteins in advanced stages of cancer progression (Esposito et al. 2006).

E. 4) Cathepsin and AnxA2

Cathepsin B is a cysteine protease and reported to be upregulated in a variety of tumors, particularly at the invasive edges. Cathepsin B is known to degrade extracellular matrix proteins, such as laminin and Collagen IV and is known to activate the precursor form of urokinase plasminogen activator (uPA), and initiating an extracellular proteolytic cascade. Cathepsin B was first associated with human and murine malignancies. Cathepsin B has been found to be significantly higher in malignant breast tissues than non-malignant breast tissues (Sloane et al. 2005) as well as in variants of the B16 melanoma with high metastatic potential (Sloane et al. 2005) in colon carcinomas (Sloane et al. 2005) and glioblastomas (Sloane et al. 2005). Mai et al. had previously demonstrated that procathepsin B interacts with the AnxA2 heterotetramer (AIIt) on the surface of tumor cells (Mai et al. 2000). The AIIt binding site for cathepsin B differs from that for either plasminogen/plasmin or tPA. Cathepsin B, tenascin-C and plasminogen/plasmin and t-PA have all been linked to tumor development and Mai et al. speculated that the colocalization of AIIt, cathepsin B may facilitate: (1) activation of precursor forms of proteases

and initiation of proteolytic cascades; and (2) selective degradation of extracellular matrix proteins. They believe that “recruitment of proteases to specific regions on the cell surface, regions where potential substrates are also bound, could well function as a 'proteolytic center' to enhance tumor cell detachment, invasion and motility”. (Mai et al. 2000)

E. 5) Exocytosis

It has been reported that secretory granules can be transported to the cell periphery which can then fuse with the plasma membrane to release their contents into the extracellular milieu in response to appropriate stimulus (Chasserot-Golaz et al. 1996). Interestingly, AnxA2 has been reported to be involved in the Ca^{2+}-dependent exocytosis of secretory granules in adrenal chromaffin cells (Chasserot-Golaz et al. 1996). The exact mechanism is still unknown but it has been shown that AnxA2 can form huge dense complexes with the plasma membrane and secretory vesicles to increase the density of the fusion complexes leading to the formation of multiple pores during membrane fusion (Faure et al. 2002). This results in membrane destabilization leading to membrane disruption and release of membrane fragments.

E. 6) AnxA2 and the exosome connection: Valapala et al. had shown that AnxA2 is secreted in the exosomes in a sequential manner (Valapala and Vishwanatha 2011). AnxA2 is recruited to the cholesterol and sphingolipid rich lipid raft regions of the membrane followed by budding of the plasma membrane rafts into the exosomal membrane. Binding of AnxA2 to the lipid rafts is followed by its transport along the endocytic pathway to be associated with the intralumenal vesicles of the multivesicular endosomes. AnxA2-containing multivesicular endosomes fuse directly with the plasma membrane resulting in the release of the intralumenal vesicles into the extracellular environment facilitating the exogenous transfer of AnxA2 from one cell to another;

a novel pathway of extracellular transport of AnxA2. (Fig. 7). Furthermore, AnxA2 has been implicated in the biogenesis of the MVBs and the exosomes (Thery et al. 2002). Interestingly, proteomic profiling of exosomes have found AnxA2 to be one of the most abundant proteins in the exosomes (Table.3) (Simpson et al. 2012). A recent study by Jeon et al. compared the relative expression of AnxA2 in the media with the invasiveness of the breast cancer cells and found that secreted AnxA2 levels correlate positively with the breast cancer invasiveness. (Jeon et al. 2013)

Fig.7

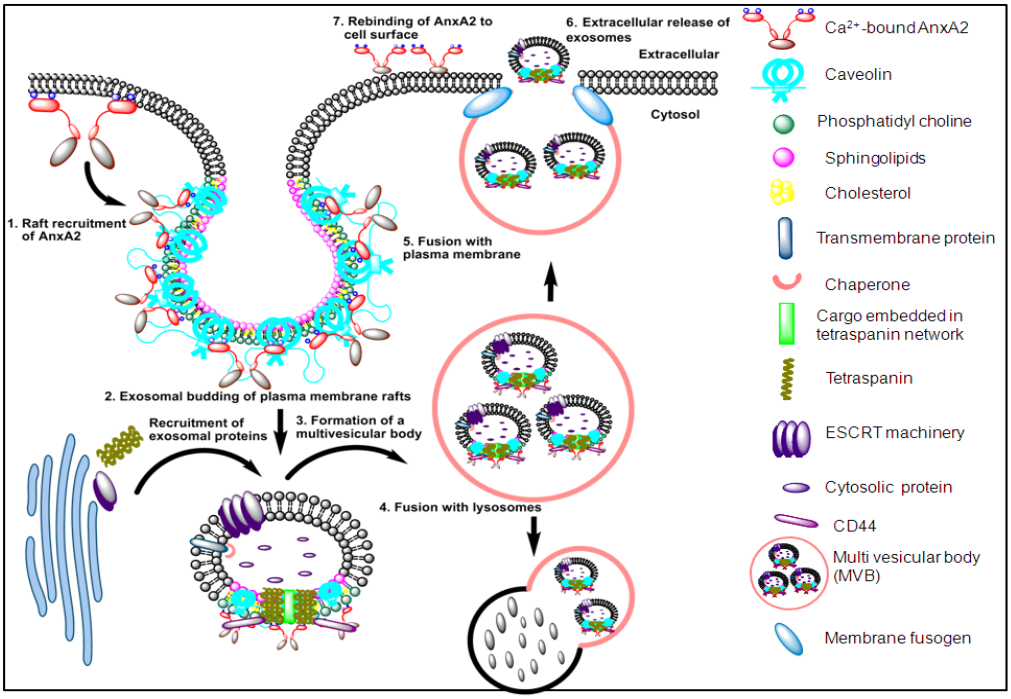

Figure 7. Extracellular secretion of AnxA2 via the exosomal pathway.

A diagrammatic representation of several steps involved in the extracellular trafficking of AnxA2. 1. Recruitment of AnxA2 to the cholesterol and sphingolipid rich lipid rafts. 2. Budding of the plasma membrane rafts into the exosomal membrane. 3. Maturation of the exosomes into a MVB by deposition of inner limiting membrane. 4. Targeting of the MVBs either for lysosomal degradation or 5. Fusion with the plasma membrane. 6. Extracellular release of the exosomes and

shedding of the exosomal contents. 7. Release of exosomal AnxA2 and binding to the cell surface.

Number	Gene Symbol	Number of times identifie
1	HSPA8	52
2	CD9	50
3	GAPDH	48
4	ACTB	43
5	CD63	41
6	CD81	39
7	ANXA2	37
8	ENO1	36
9	HSP90AA1	34
10	EEF1A1	34

Table. 3

Table 3. List of most commonly expressed exosomal proteins. Source: Exocarta

Central Hypothesis and Rationale:

Our central hypothesis is "*Breast cancer derived exosomal AnxA2 promotes angiogenesis and creates a suitable microenvironment that promotes breast cancer metastasis.*" Our hypothesis is based on the rationale that AnxA2 is an important protein component of the exosomes – (Simpson et al. 2012) and is one of the most commonly expressed proteins in the exosomes. Further, we have seen that once secreted exosomal AnxA2 is successfully taken up by the recipient cells (Valapala et al. 2011) and we believe that exosome mediated transfer of AnxA2 is an important mechanism by which AnxA2 elicits its function. A recent study by Jeon et al. showed that secreted AnxA2 levels positively correlate with the aggressiveness of cancer cells (Jeon et al. 2013). Based on this we believe that exosomal AnxA2 promotes angiogenesis and increased tumor-stroma signaling creating a microenvironment promoting metastasis.

We believe that cancer cells secrete exosomal AnxA2 which is taken up by recipient cells. Once inside, exosomal AnxA2 promotes angiogenesis and cancer metastasis. Thus, we believe that exosomal AnxA2 is an important component of the tumor – microenvironment signaling (Fig. 8). We studied the role of exosomal AnxA2 is breast cancer pathogenesis since AnxA2 has been implicated in poor breast cancer prognosis (Lokman et al. 2011). Furthermore, breast cancer has well established progression and metastatic models which provides an ideal setup to characterize exosomal AnxA2 and delineate its role in angiogenesis and metastasis.

We tested our hypothesis under three specific aims:

Specific Aim 1: To characterize of exosomal AnxA2 in a breast cancer progression model.

Specific Aim 2: To determine the role of exosomal AnxA2 in angiogenesis and breast cancer metastasis.

Specific Aim 3: To validate whether serum exosomal AnxA2 from breast cancer patients can be developed as a potential biomarker.

Specific aim 1 was studied in chapter II, specific aim 2 was studied in chapter III and IV and specific aim 3 was studied in chapter V.

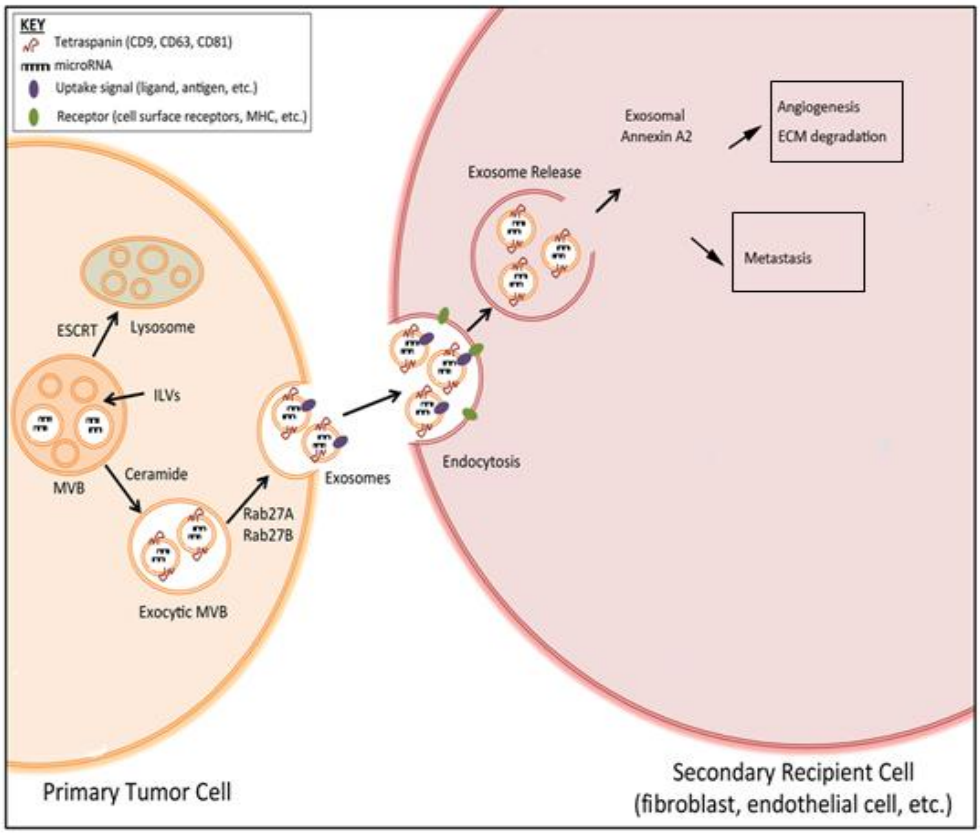

Figure 8. Proposed model for mechanism of action of exosomal AnxA2

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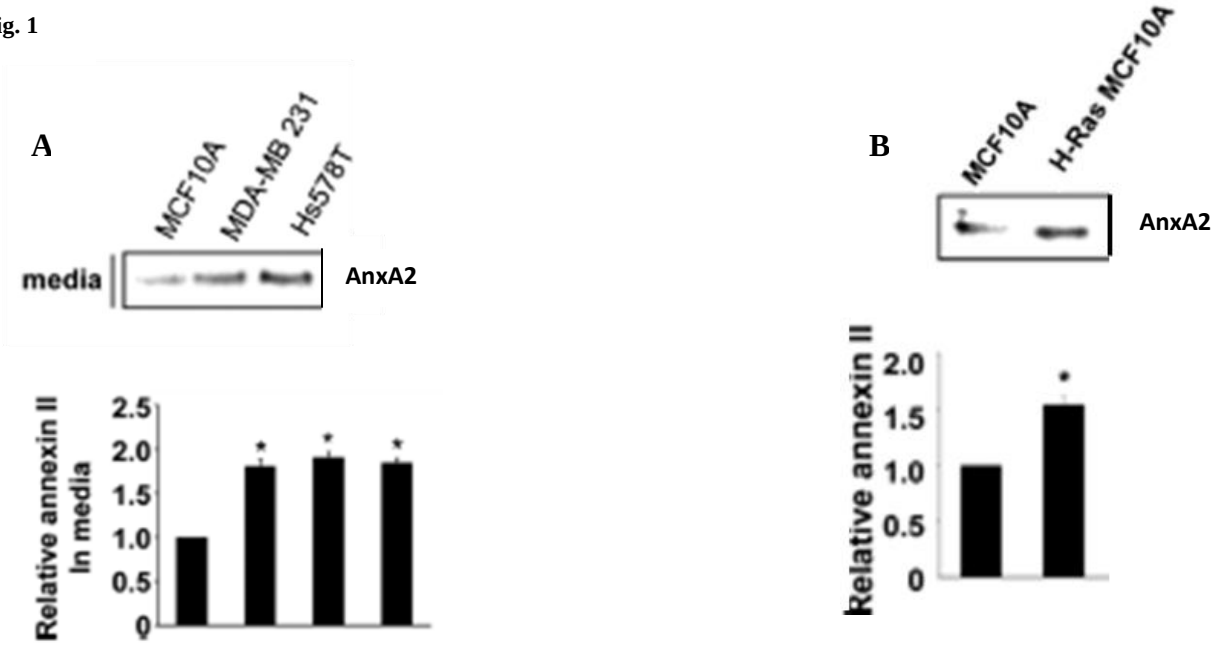
CHAPTER II. CHARACTERIZATION OF EXOSOMAL ANNEXIN A2

Introduction:

As mentioned earlier, AnxA2 is one of the most commonly expressed proteins in the exosomes (Simpson et al. 2012). AnxA2 has also been reported to be involved in early endosome fusion and in fusion of secretory granules with the plasma membrane. (Chasserot-Golaz et al. 2005). A recent article by Jeon et al. showed that AnxA2 was secreted more efficiently in invasive breast carcinoma cells compared to noninvasive cell lines. They also found that secreted levels of AnxA2 were higher in Hs578T and MDA-MB-231 TNBC cell lines, suggesting that AnxA2 can be a potential target for diagnosis and/or therapy of TNBC. Proteomic profiling of the secreted AnxA2 levels showed that there is relative increase of AnxA2 levels in the media of H-Ras MCF10A (MCF10A cells with oncogenic H-Ras transformation) cells compared to the parental MCF10A cells (Jeon et al. 2013) (Fig. 1). Both Valapala et al. and Jeon et al. reported that AnxA2 is secreted through an unconventional pathway and they further demonstrated that AnxA2 secretion plays an important role in invasive phenotype of H-Ras MCF10A cells. They also discovered that plasma AnxA2 levels breast cancer patients were significantly higher than those in normal group (Valapala and Vishwanatha 2011, Jeon et al. 2013).

In this study, we characterized and correlated exo-AnxA2 levels in the MCF10A breast cancer progression model which consists of MCF10A cells (non-malignant), MCF10AT (pre-malignant) cells and MCF10CA1a (malignant) breast cancer cells to have a better understanding of the possible role of exo-AnxA2 in breast cancer progression and tumorigenesis.

Fig. 1



Jeon et al. Proteomics, 2013

Figure 1. Correlation of secreted levels of AnxA2 and invasive abilities of cell lines. (A) Protein level of AnxA2 in conditioned media as detected by immunoblot analysis in invasive cell lines MDA-MB-231, Hs578T and a noninvasive cell line, MCF10A (*, $p < 0.01$). (B) Increased level of AnxA2 in media of H-Ras MCF10A cells. The protein level of AnxA2 in media as examined by immunoblot analysis.

Materials and Methods:

Cell line models: For the comparative analysis of exo-AnxA2, the MCF10A progression model was used: MCF10A (non-malignant), MCF10AT (pre-malignant) and MCF10CA1a (malignant). (Received as a gift from Dr. Judith Christman, UNMC). MCF10A (Debnath et al. 2003) and MCF10AT, MCF10CA1a (Lee et al. 2007) cells were cultured according to previously published literature. Briefly, MCF10 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) F12 media along with L-Methionine, Calcium Chloride, Sodium Bicarbonate, horse serum, hydrocortisone, insulin Cholera toxin and Epidermal Frowth Factor (EGF) as supplements.

Antibodies and reagents: The following antibodies were used: anti-AnxA2 (BD Biosciences), anti-heat shock cognate protein-70 (Hsc-70) (Enzo), anti-GAPDH, anti-CD81 (Santa Cruz Biotechnologies), VEGF (Cell Signaling Technologies), calnexin (Enzo), CD63 (Developmental

Studies Hybridoma Bank, Univ. of Iowa), uPA (R & D Biosystems), 25 nm gold nanoparticle tagged secondary anti-mouse antibody (ElectronMicroscopySciences),

Exosome isolation and characterization: Exosomes were isolated by ultracentrifugation according to previously published protocol (Lasser et al. 2012). Briefly, the conditioned media was centrifuged at 300 x g for 10 mins at 4 °C to pellet the cells. Next, the supernatant was transferred to ultracentrifuge tubes and centrifuged at 16,500 x g for 20 mins at 4 °C to further remove cells and cell debris. The supernatant was filtered through a 0.2 μm filter to remove particles larger than 200 nm. Finally, the filtered supernatant was transfered to new ultracentrifuge tubes and ultracentrifuged at 120,000 x g for 70 mins at 4 °C to pellet the exosomes. The supernatant was discarded and the pellet was resuspended in appropriate buffer (PBS or lysis buffer).

Size analysis of exosomes: Average particle sizes of the nanoparticles were determined by Nanotrac Particle Size Analyzer (Mircotrac, Inc., Montgomeryville, PA). The exosomal pellet was resuspended in PBS and the size distribution was analyzed. The results were reported as the average of five runs with triplicates in each run.

Quantification of exosomes: Quantification of secreted exosomes was performed by measuring the activity of acetylcholine esterase as previously described (Valapala and Vishwanatha 2011, Merendino et al. 2010). Briefly, 50 μl of the pelleted exosomes was suspended in 100 μl of PBS; 37.5 μl of this PBS-diluted fraction was incubated in the presence of 1.25 mM acetylthiocholine and 0.1 mM 5′m5′-dithiobis(2-nitrobenzoic acid), and the reaction mixture was brought to a final volume of 300 μl in a 96-well plate. The incubation was carried out at 37 °C, and the change in absorbance at 412 nm was monitored every 5 mins. The data presented represent the enzymatic activity 20 mins after incubation.

Electron microscopy: Electron microscopy of the exosomes was performed according to previously published protocol. (Lasser et al. 2012) Briefly, the PBS resuspended exosomal pellet was further centrifuged at 120,000 x g for 70 mins at 4 °C to re-pellet the exosomes. A small aliquot of the sample was taken for protein isolation and total protein measurement. A small drop containing approximately 10 μg of the intact exosomes resuspended in PBS was placed on a parafilm. Then a formvar carbon coated nickel grid was gently positioned on top of each drop for 30-60 minutes and then washed with PBS thrice. The samples were post-fixed with 2.5% glutaraldehyde for 10 minutes, contrasted by adding 2% uranyl acetate and then embedded by addition of 0.13% methyl cellulose and 0.4% uranyl acetate for 10 mins. The sample was examined with an LEO EM910 electron microscope.

Atomic Force Microscopy (AFM) of exosomes: AFM of exosomes was performed as published previously. Briefly, purified exosomes were diluted 1:100 in de-ionized water and adsorbed to freshly cleaved mica sheets, rinsed with de-ionized water and dried under a gentle stream of Nitrogen. (Sharma et al. 2010) Next, they were incubated with 1:100 dilution of monoclonal anti-AnxA2 antibody, followed by 20 mins incubation with 1:1000 dilution secondary anti-mouse antibody coated 10nm gold particles (Electron Microscopy Sciences) with consecutive PBS washes after each binding step. (Sharma et al. 2010) The exosomes were analyzed by a scanning microscope of Prima Bio series (NT-MDT, Russia) equipped with silicon test probe, with a characteristic stiffness of 5.5 N/m and a typical radius of curvature of the tip (less than 10 nm). The initial amplitude of scanning was set to 16 nA in current terms; Set Point was adjusted to half of the amplitude. Scanning was performed with a frequency of 1.01 Hz. The images were processed using standard software package (Image Analysis Nova).

Statistical Analysis

Results were expressed as arithmetic means ± SEM if not otherwise indicated. Values of $P \leq 0.05$ were considered statistically significant, as determined by the unpaired Mann–Whitney test, the two-tailed unpaired Student *t* test, or the Wilcoxon signed rank test where appropriate. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ for all the figures. Statistical software GraphPad Prism was used for all statistical analysis.

Results:

Exosome isolation and characterization:

Successful exosome isolation was confirmed by transmission electron microscopy (TEM) and size analysis. TEM analysis of the isolated exosomes from breast cancer cells showed the typical cup shaped exosome structure (Fig. 2, left and middle panels). Size analysis of the exosomes by NanoTrac particle size analyzer revealed that exosomes were 40-110 nm in diameter with an average size of 61.7 nm (Fig. 2, right panel). On quantification of the amount of exosomes released along the MCF10A progression model via acetylcholine esterase assay and found that MCF10CA1a cells secrete ~1.8 and ~3fold more exosomes than MCF10AT or MCF10A respectively, confirming that cancer cells secrete more exosomes than normal or non-malignant cells as previously suggested (Kharaziha et al. 2012) (Fig. 3).

Fig. 2

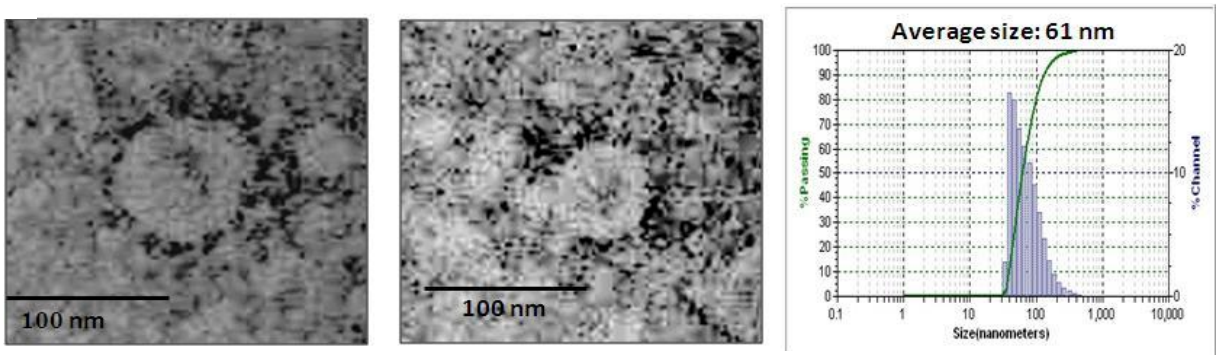


Figure. 2: TEM analysis of exosomes from MCF10CA1a and MDA-MB-231 cells (left and middle panels). Size analysis of the exosomes (right panel)

Fig. 3

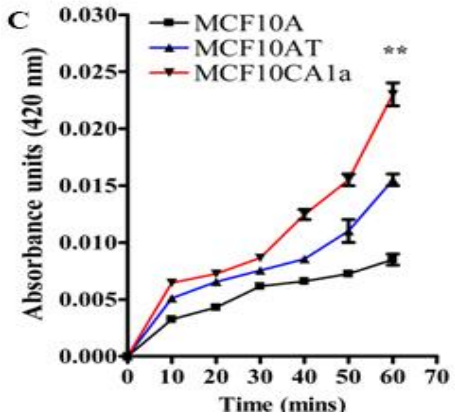


Figure. 3: Exosome release from MCF10 progression model as quantified by acetylcholine esterase assay.

P < 0.001, *
**P < 0.01, **
P < 0.05, *

Exo-AnxA2 correlates positively with the aggressiveness of breast cancer cells. To characterize exo-AnxA2 expression, we used the MCF10A breast cancer progression model which consists of MCF10A (non-malignant), MCF10AT (pre-malignant) and MCF10CA1a (malignant) cells. Western immunoblotting of exosomes from MCF10A, MCF10AT and MCF10CA1a revealed that exo-AnxA2 levels highly correlate with the aggressiveness of the breast cancer cells, with lower levels in MCF10A, moderate in MCF10AT and in significantly higher levels in MCF10CA1a (Fig. 4 A); while the whole cell lysate (WCL) analysis of the progression model revealed no significant changes in the levels of AnxA2 in MCF10AT and MCF10CA1a (Fig. 4 D). Densitometric analysis revealed ~5 fold increase in exo-AnxA2 levels in MCF10CA1a exosomes than MCF10A exosomes (Fig. 4 B). The levels of other angiogenic markers including Vascular Endothelial Growth Factor (VEGF) and Urokinase-type Plasminogen Activator (uPA) as well as matrix metalloproteinase 9 (MMP9) were relatively unchanged. CD81 was used as a specific exosomal marker and as loading control. The purity of the exosomal fraction was confirmed by immunoblotting the exosomes collected from the conditioned media with antibody against endoplasmic reticulum marker (calnexin) as negative control (Fig. 4 C). We found Hsc-70 to be highly expressed in MCF10CA1a exosomes but almost negligible in MCF10A and

MCF10AT exosomes (Fig.4 A). Interestingly, knockdown of AnxA2 expression in the exosomes also leads to Hsc-70 down-regulation in the exosomes (Fig. 4 E).

Fig. 4

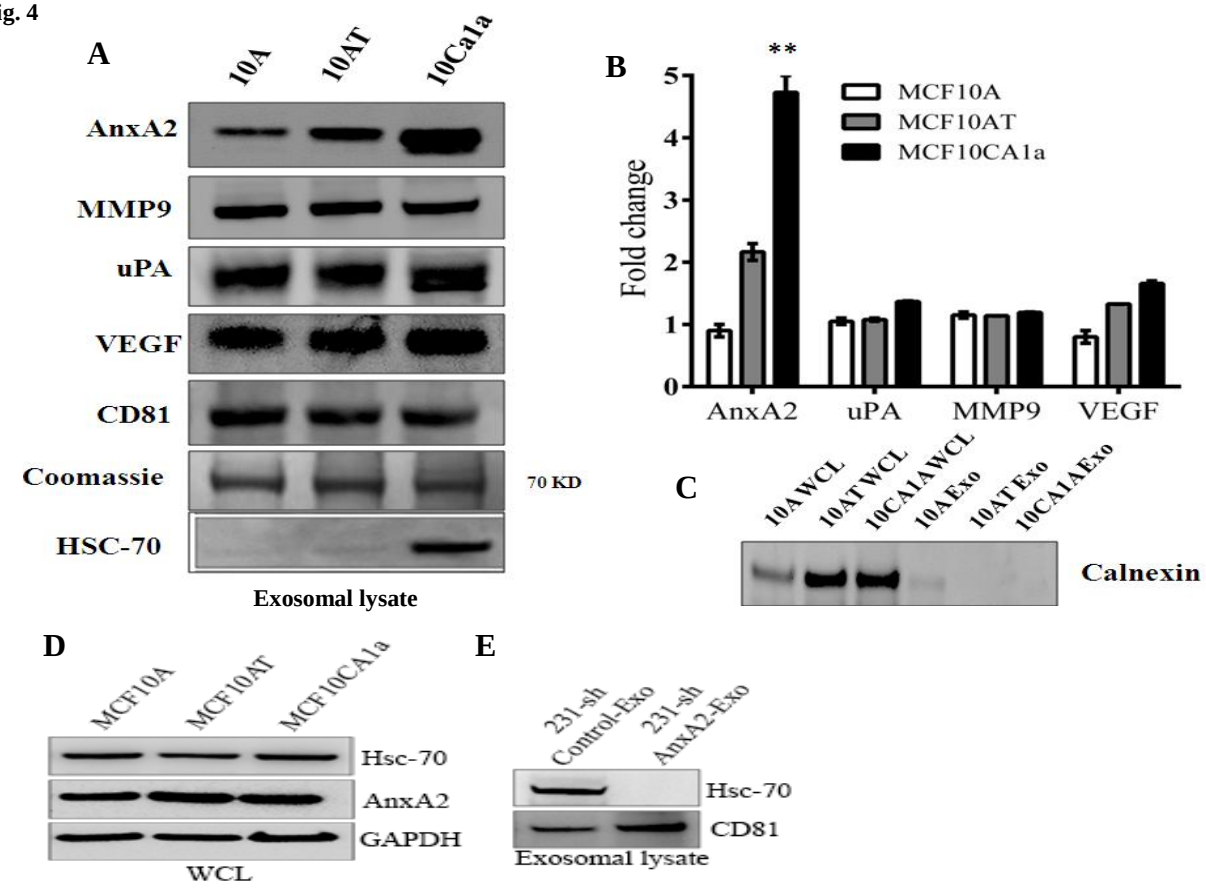


Figure 4: ***Characterization of exosomal AnxA2*** (A-F). A) Western blot analysis of the different protein levels in the exosomes collected from the MCF10A, MCF10AT and MCF10CA1a conditioned media. Exosome enriched tetraspanin family protein CD81 was used as a loading control (n=3). 70kDa coomassie band was shown to confirm equal loading. B) Quantification of the Western blots. Fold change to CD81 is shown. C) Endoplasmic reticulum (ER) protein Calnexin was used as a negative control for exosomes. Western Blot showing the purity of the exosomal fractions. D) Western blot analysis of Hsc-70 and AnxA2 in the WCL of MCF10A progression model. GAPDH was used as a loading control. E) Western blot analysis of Hsc-70 in

the exosomal lysate of MDA-MB-231-shControl-Exo and MDA-MB-231-shAnxA2-Exo. CD81 was used as a loading control.

Surface expression of exo-AnxA2 is higher in MCF10CA1a exosomes than MCF10A exosomes: Scanning of exosomes in the MCF10A progression model by Atomic Force Microscopy (AFM) revealed that AnxA2 was located on the exosomal outer membrane. Interestingly, AFM analysis revealed a higher surface expression of AnxA2 in MCF10CA1a exosomes than MCF10A exosomes as depicted by increased surface binding of 25 nm gold nanoparticle tagged anti-AnxA2 antibody (Fig. 5 A). Quantification of the number of gold nanoparticles per field revealed almost ~5fold more binding of gold nanoparticles on MCF10CA1a exosomes than MCF10A exosomes (Fig. 5 B). Surface topography analysis confirmed these findings showing multiple peaks corresponding to higher gold nanoparticle binding in MCF10CA1a exosomes with CD63 and AnxA2 antibody treatments, but much lesser in MCF10A and MCF10CA1a exosomes with AnxA2 antibody and CD63 antibody treatment respectively (Fig. 5 C). This indicates that MCF10CA1a exosomes have more AnxA2 surface expression than MCF10A exosomes. CD63 and Calnexin served as positive and negative controls respectively.

Fig. 5

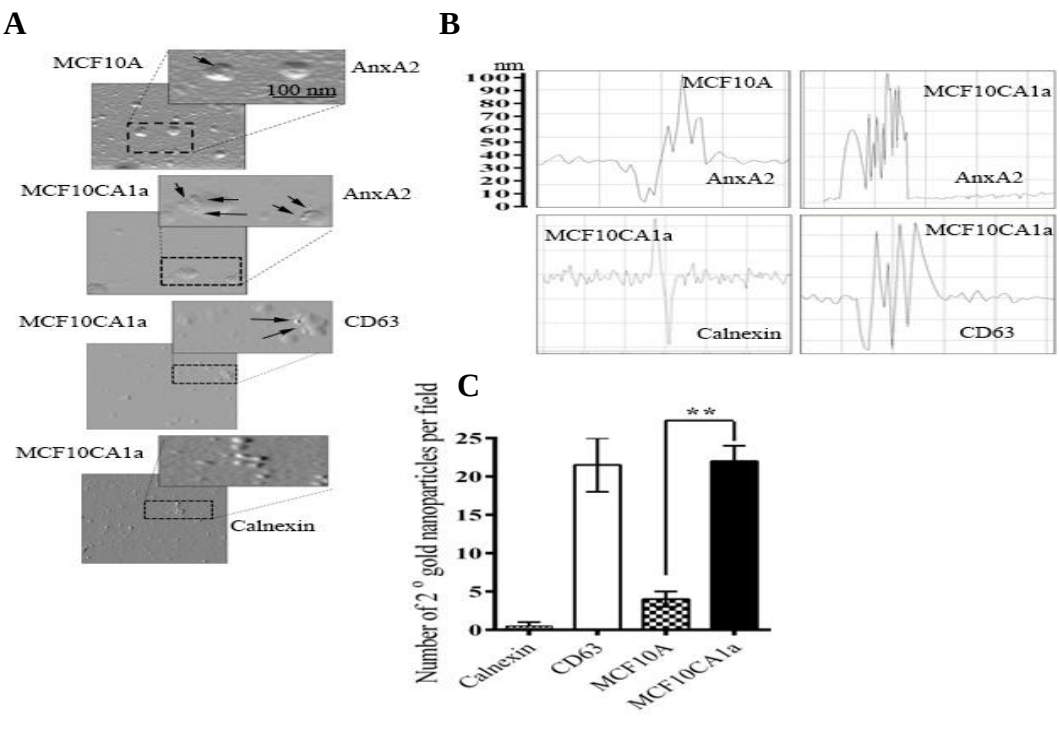


Figure. 5: A) AFM analysis of MCF10A exosomes and MCF10CA1a exosomes. Exosomes with incubated with respective AnxA2, Calnexin and CD63 primary antibodies followed by incubation of 25nm gold nanoparticles tagged secondary antibodies. Higher binding of gold nanoparticles indicate higher surface expression of the target proteins. B) Quantification of the AFM data representing the number of secondary gold nanoparticles per field. Four independent fields were counted. C) Surface topology analysis of the exosomes using the AFM NT-MDT software (n=2).

Discussion:

The current study explores an important correlation of an abundant exosomal protein – AnxA2 in a breast cancer progression model. We successfully isolated exosomes from the cell culture supernatant as established by the TEM and size analysis data. Detailed Western blot and AFM analysis of the exosomes in the MCF10 progression model showed that exo-AnxA2 levels positively correlate with the aggressiveness of breast cancer as previously suggested (Jeon et al. 2013); where as the expression levels of other pro-angiogenic proteins were unaltered. This supports our hypothesis that exo-AnxA2 is an important component of cancer exosomes and play important roles in breast cancer pathogenesis.

Interestingly, we found Hsc-70 (a widely accepted exosomal marker) (Meckes et al. 2010) to be highly expressed in MCF10CA1a exosomes but almost negligible in MCF10A and MCF10AT exosomes. Knockdown of AnxA2 expression in the exosomes also leads to Hsc-70 down-regulation in the exosomes indicating that AnxA2 is upstream of Hsc-70 and AnxA2 might play an important role in exosome biogenesis and packaging as proposed by Thery et al. (Thery et al. 1999) owing to the fact that Hsc-70 plays a key role in exosome formation and/or

release. Interestingly, McCready et al. reported that another heat shock protein Hsp-90α is a part of an extracellular complex including AnxA2, tPA and plasminogen that functions to increase cell movement (McCready et al. 2010). These reports and our data indicate an important link between exo-AnxA2 and Hsps and their possible role in the cancer pathogenesis.

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CHAPTER III. ROLE OF EXOSOMAL ANNEXIN A2 IN ANGIOGENESIS

Introduction:

For the endothelial cells to migrate, proliferate and form new blood vessels, extracellular proteolysis is an indispensable process (Pepper 2001, van Hinsbergh et al. 2006). The extracellular proteolytic system comprises of serine proteases as well as their activators including urokinase plasminogen activator (uPA), tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2) (Lijnen 2001). Plasmin which is a broad spectrum trypsin-like serine protease that degrades several components of the ECM like laminin and fibrin, activates inactive pro-matrix metalloproteinases (MMPs) and also increases the bioavailability of VEGF165 and VEGF189 is a central component of this system (Hajjar et al., Lijnen 2001). Plasmin-induced angiogenic response plays an important role in various disease processes including but not limited to diabetic retinopathy and cancer (Simpson et al.). AnxA2 is a cell surface co-receptor for tPA and plasminogen (Kim et al. 2002). AnxA2 binds to both plasminogen and tPA and brings the two enzymes in close proximity thereby increasing the catalytic efficiency of plasmin generation by ~60fold (Hajjar et al. 1997). This results in the initiation of a highly efficient plasmin-mediated proteolytic cascade which promotes angiogenesis by increasing the efficiency of endothelial cell invasion across the ECM (Hajjar and Acharya 2000).

tPA reportedly binds to the N-terminus of AnxA2 corresponding to the site LCKLSL encompassing the 7-12 residues of AnxA2 (Cesarman et al. 1999, Roda et al. 2003). The presence of cysteine at position 8 is critical for the tPA binding activity and the mutation of this residue significantly reduces the binding efficiency of tPA to AnxA2 (Roda et al. 2008). Previously it has been shown that a hexapeptide with the sequence LCKLSL that is identical to

the tPA-binding site of AnxA2 and containing a cysteine residue at position 8 is more than 95% efficient in competitively inhibiting tPA binding to AnxA2 (Roda et al. 2008).

In this report, we studied the angiogenic activity of exo-AnxA2. By using various *in vitro* and *in vivo* models, we showed that exo-AnxA2 is a potent inducer of angiogenesis. Furthermore, using the LCKLSL inhibitory peptide and the LGKLSL control peptide we showed that treatment with the competitive peptide inhibitor prevents exo-AnxA2 mediated angiogenesis. However, no significant change was found with the LGKLSL control peptide, when compared to exosome treatment only. We also confirmed our findings by down regulating AnxA2 levels in the exosomes. Taken together, this study elucidates how an exosomal protein can play an important role in angiogenesis.

Materials and Methods:

Peptide synthesis: The LCKLSL and control LGKLSL peptides were synthesized using solid phase methods (Genscript). The peptides were purified (≥ 94%) by preparative reverse phase HPLC. The hexapeptide sequence was analyzed by MALDI-TOF mass spectrometry. Biotinylated peptides were prepared by adding biotin groups to the C-terminal lysine residue of the peptides by an amide bond. The positive charge on the lysine residue was later removed.

In vitro migration and invasion assay: Migration and invasion assay was performed with transwell invasion assay inserts (BD Biosciences) and 24-well plates according to manufacturer's protocol. Briefly, 25,000 HUVEC cells (in triplicates) were placed in the upper chamber of the transwell inserts in a total volume of 500 µl serum free media mixed with PBS/ 10CA1a exosomes/ 10CA1a exosomes + LCKLSL (5 µM) or 10CA1a exosomes +LGKLSL (5 µM) and kept overnight. Fetal bovine serum was added to the lower chamber as a chemo attractant. The inserts were coated with 100 µl of matrigel (BD Biosciences) for invasion assay

or without matrigel for migration assay. After 24 hrs the lower side of the transwell membranes were fixed and stained with 0.05% crystal violet. For migration, the cells have to pass through the membrane pore and reach the other side of the membrane, whereas in case of invasion, the cells will first have to invade through the matrigel and then pass through the membrane pore to reach the other side of the membrane. The number of cells migrating (without matrigel) and invading (through the matrigel) was calculated. Number of cells migrating or invading with just 10CA1a treatment was considered as 100% and percent change to 10CA1a treatment was calculated (n=3).

In vitro endothelial tube formation assay: *In vitro* endothelial tube formation assay was performed according to previously published literature with slight modifications. (Umezu et al. 2013). Growth factor reduced BD Matrigel (BD Biosciences) (0.2 mL each) was added to 24-well dishes and placed in a humidified CO_2 incubator at 37°C for 2 hrs, allowing the gel to solidify. Passage 4 or passage 5 subconfluent HUVECs were harvested and resuspended in growth factor reduced medium and treated with PBS, VEGF (100 ng/ml) or exosomes (100 µg/ ml). This suspension was seeded in growth factor reduced matrigel-coated 24 well plates (final concentration 2×10^4 cells/mL) and incubated up to 6 hrs at 37 degree C in the CO_2 incubator. At the end of the incubation period after the tube network had formed, the cells were stained with Calcein-AM cell permeable dye for 30 mins at 37°C and 5% CO_2 (protected from light). Final dye concentration used was 2 µg/mL. After incubation, the dye containing medium was removed, washed with PBS and images were taken from 6 independent fields per well using a Zeiss Axiovert 40 inverted fluorescence microscope. To validate that the angiogenic effect was mainly due to exosomal AnxA2, we used AnxA2 specific inhibitor peptide LCKLSL or control peptide LGKLSL. (Valapala et al. 2011) Tube lengths and tube areas and branch points will be

analyzed via the angiogenesis analyzer plugin of the Image-J software.

In vivo matrigel plug assay: The Matrigel plug assay was performed as described previously (Merchan et al., 2010) with slight modifications. Briefly, 500 µl of unpolymerized growth factor reduced high concentration Matrigel (BD Biosciences) (~20 mg/ml), either with PBS (negative control), mixed VEGF (100 ng/ml each), or in the presence of different exosome treatments with or without LGKLSL/ LCKLSL peptides was injected subcutaneously at the left or right lower abdominal wall of athymic nude mice (4- to 6-weeks-old) (Harlan Laboratories, Madison, WI). Mice were killed 16-18 days after the Matrigel injections, and the plugs were recovered, photographed. Half of the plugs per group were fixed in formaldehyde and embedded in paraffin for sectioning. Rests of them were snap frozen in liquid nitrogen for hemoglobin estimation.

Hemoglobin estimation by Drabkin's reagent: Hemoglobin estimation from the matrigel was performed by Drabkin's method. **(**Drabkin et al. 1935) To quantify the formation of functional vasculature in the Matrigel plug, the amount of hemoglobin was measured using a Drabkin reagent kit 525 (Sigma, St. Louis, MO) following the Drabkin and Austin method. Briefly, the matrigel plugs were chopped and homogenized in a Dounce homogenizer on ice in presence of 0.5 ml deionized water and allowed to stand overnight at 4 ºC. The lysate was centrifuged at 5000 x g for 10 mins and the supernatant was collected. 0.3 ml of each sample was mixed with 0.5 ml of Drabkin's reagent and allowed to stand for 15 mins at room temperature. The absorbance was read at 540 nm by using Drabkin's reagent solution as blank. A standard curve was constructed by using known concentrations of hemoglobin and the concentrations of the samples were obtained from the standard curve.

Statistical Analysis: Results were expressed as arithmetic means ± SEM if not otherwise indicated. Values of $P \leq 0.05$ were considered statistically significant, as determined by the

unpaired Mann–Whitney test, the two-tailed unpaired Student *t* test, or the Wilcoxon signed rank test where appropriate. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ for all the figures. Statistical software GraphPad Prism was used for all statistical analysis.

Results:

Exo-AnxA2 promotes angiogenesis: AnxA2-p11-tPA mediated plasmin generation is known to be a potent inducer of angiogenesis (Lokman et al. 2011, Hajjar and Acharya 2000). We investigated whether exo-AnxA2 also is capable of promoting angiogenesis once secreted from the cells. We used LGKLSL control peptide and AnxA2 specific inhibitory peptide LCKLSL (which blocks the tPA binding site of AnxA2) to specifically inhibit the function of exo-AnxA2 (Valapala et al. 2011). Collected exosomes (100 µg) from cells of MCF10A progression model were incubated (with/without LCKLSL or LGKLSL peptide) with HUVEC endothelial cells plated on matrigel and tube formation assay was performed. *In vitro* endothelial tube formation assay with MCF10A, MCF10AT and MCF10CA1a exosome treatments showed ~4fold, ~6fold and ~7.5fold increase in the number of meshes per field (Fig. 1 B) and ~1.4fold, ~1.8fold and ~2 fold increase in the number of branch points per field vs. PBS treatment respectively (Fig. 1 D). Incubation of MCF10CA1a exosomes with LCKLSL nullified the angiogenic effect of exo-AnxA2 vs. exosome treatment only or with LGKLSL treatment which is evident from the decrease in the number of meshes per field ~3fold (Fig. 1 C) and the number of branch points per field ~0.75fold (Fig. 1 E). Treatment with MCF10A and MCF10AT exosomes plus LCKLSL also had similar effects (Fig. 2).

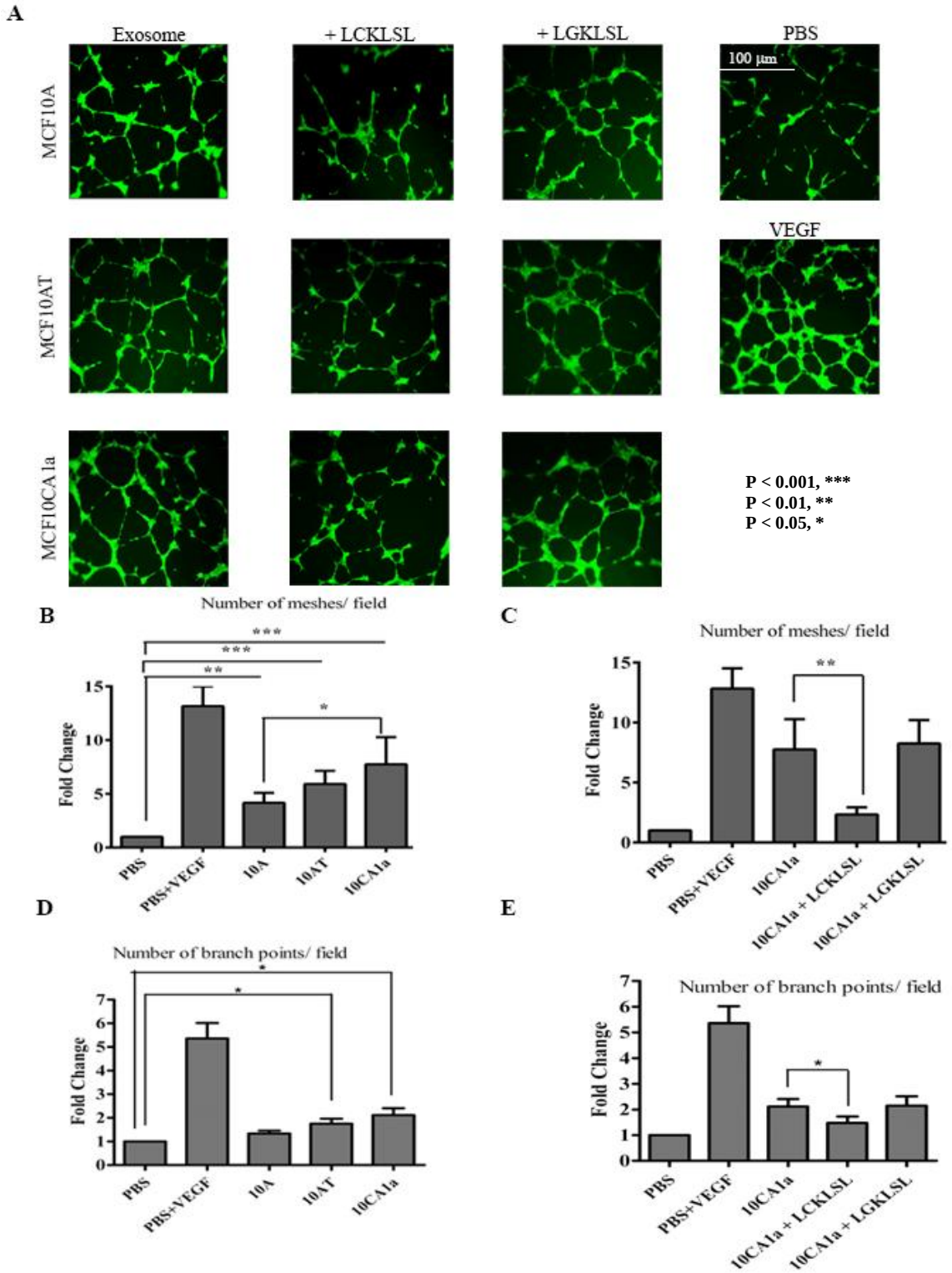
Endothelial cell migration and invasion is an important step in angiogenesis (Kumar et al. 1998) and AnxA2-p11-tPA mediated plasmin generation is known to be a potent promoter of this process (Lokman et al. 2011). Thus, to further confirm the role of exo-AnxA2 in plasmin

generation, ECM degradation and angiogenesis, we studied the effect of exo-AnxA2 on migration and invasion of HUVEC cells. HUVEC endothelial cells were incubated overnight with different exosome treatments with or without LCKLSL or LGKLSL. Treatment with MCF10CA1a exosomes plus LCKLSL led to a significant decrease in both migration (~40%) and invasion (~30%) of HUVEC cells in transwell migration and invasion assays than MCF10CA1a exosomes or MCF10CA1a exosomes plus LGKLSL treatments (Fig. 3 A-C).

To confirm our *in vitro* findings in an *in vivo* angiogenesis model, we used the matrigel plug assay (Merchan et al. 2010). Subcutaneous injection of matrigel with 100 μg of MCF10A, MCF10AT or MCF10CA1a exosomes induced significant angiogenesis as evident from the matrigel plug images (Fig. 3 D); MCF10CA1a exosomes showed significantly higher angiogenesis than MCF10AT and MCF10A exosomes treatments. Hemoglobin estimation of the homogenized matrigel plugs confirmed these results showing ~5fold, ~14fold and ~24fold increase in hemoglobin content when incubated with MCF10A, MCF10AT and MCF10CA1a exosomes respectively vs. PBS treatment (Fig. 3 E). Consistent with our *in vitro* findings, incubation of matrigel plugs-exosomes mixture with LCKLSL resulted in drastic decrease in angiogenesis as evident from matrigel plug images (Fig. 3 D). Hemoglobin content analysis from homogenized matrigel plugs confirmed these results showing ~5 fold decrease with LCKLSL treatment than LGKLSL or exosome treatment only (Fig. 3 F). Injection of matrigel with MCF10A or MCF10AT exosomes plus LCKLSL also had similar effects (Fig. 3 D, Fig. 4 A-B). Hematoxylin and Eosin (H & E) staining (Fig. 5 A-B) and DAB (3, 3'-diaminobenzidine) immunostaining of matrigel plug sections against endothelial marker CD31 (recruitment of CD31 positive endothelial cells indicates increased angiogenesis) (Fig. 5 C-D) confirmed our findings showing lesser CD31 positive endothelial cells in LCKLSL treatment than LGKLSL

treatment (Fig. 5 A-D). Further, H & E as well as CD31 staining also revealed a marked reduction in the number of the capillaries in the plugs with LCKLSL treatment than LGKLSL treatment. We also reconfirmed our findings by knocking down AnxA2 levels in the exosomes in a different breast cancer cell line. *In vitro* endothelial tube formation assay and *in vivo* matrigel plug assay were performed with exosomes after knocking down AnxA2 (MDA-MB-231-shAnxA2-Exo) and compared to control (MDA-MB-231-shControl-Exo). Matrigel plug assay with MDA-MB-231-shAnxA2-Exo treatment revealed ~5fold less hemoglobin per matrigel than MDA-MB-231-shControl-Exo (Fig. 6 A-B). Quantification of *in vitro* angiogenesis assay showed ~4.5fold and ~3fold decrease in the number of meshes per field and number of branch points per field respectively with MDA-MB-231-shAnxA2-Exo treatment than MDA-MB-231-shControl-Exo treatment (Fig. 6 C-D).

Fig. 1


A
Exosome
+ LCKLSL
+ LGKLSL
PBS
100 µm
MCF10A
MCF10AT
MCF10CA1a
VEGF
P < 0.001, ***
P < 0.01, **
P < 0.05, *
B
Number of meshes/ field
Fold Change
PBS
PBS+VEGF
10A
10AT
10CA1a
C
Number of meshes/ field
Fold Change
PBS
PBS+VEGF
10CA1a
10CA1a + LCKLSL
10CA1a + LGKLSL
D
Number of branch points/ field
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PBS
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10A
10AT
10CA1a
E
Number of branch points/ field
Fold Change
PBS
PBS+VEGF
10CA1a
10CA1a + LCKLSL
10CA1a + LGKLSL

Figure 1: Effect of exo-AnxA2 on in vitro tube formation assay (A-E). A) Fluorescent images of *in vitro* endothelial tube formation assay with HUVEC cells; (5 different fields per group were considered. Repeated in triplicates). PBS (negative control), VEGF (100 ng/ml, positive control) and 100 µg of exosomal proteins were used (n=3). Quantification of endothelial tube formation assay: Number of meshes per field (B-C); number of branch points per field (D-E). Peptide concentration used: 5 µM (n=3). Exosomes from MCF10A, MCF10AT and MCF10CA1a cells are designated as 10A, 10AT and 10CA1a respectively. Scale bar 100 µm.

Fig. 2

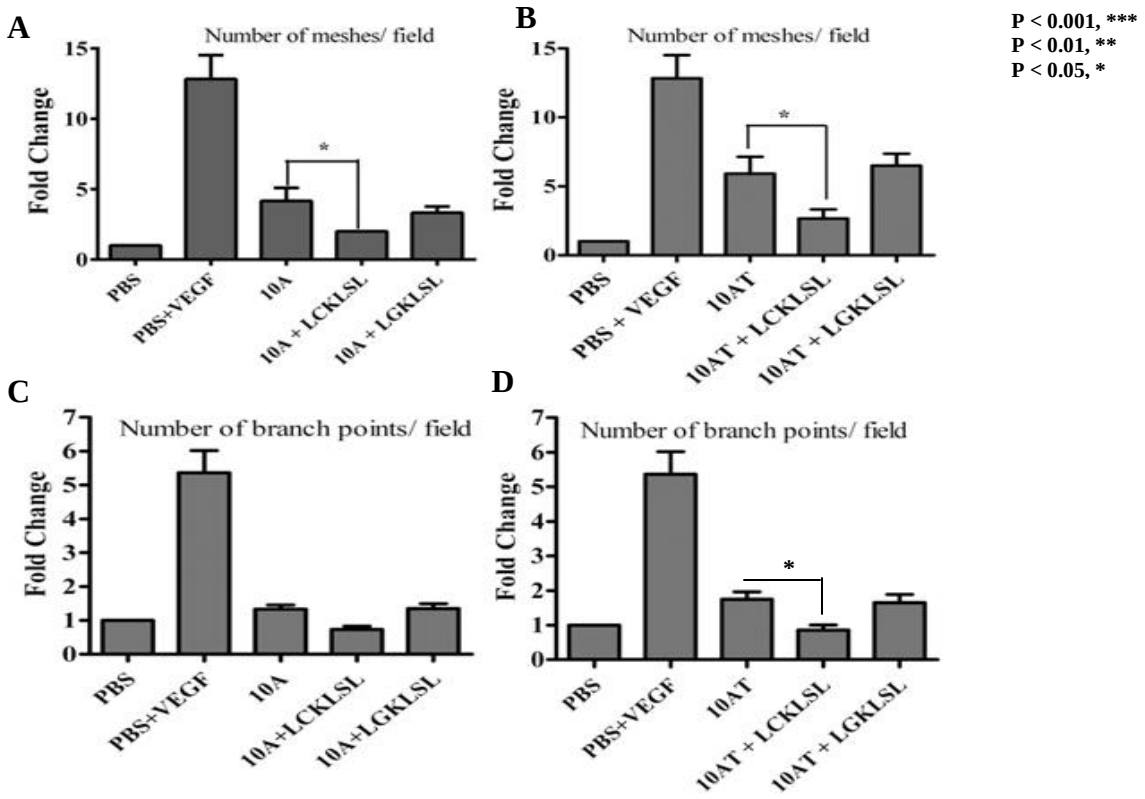

Figure 2: Quantification of tube formation assay with MCF10A and MCF10AT exosomes in presence of LCKLSL or LGKLSL peptides: number of meshes per field (A-B) and number of branch points per field (C-D). Exosomes from MCF10A, MCF10AT and MCF10CA1a cells are designated as 10A, 10AT and 10CA1a respectively.

Fig. 3

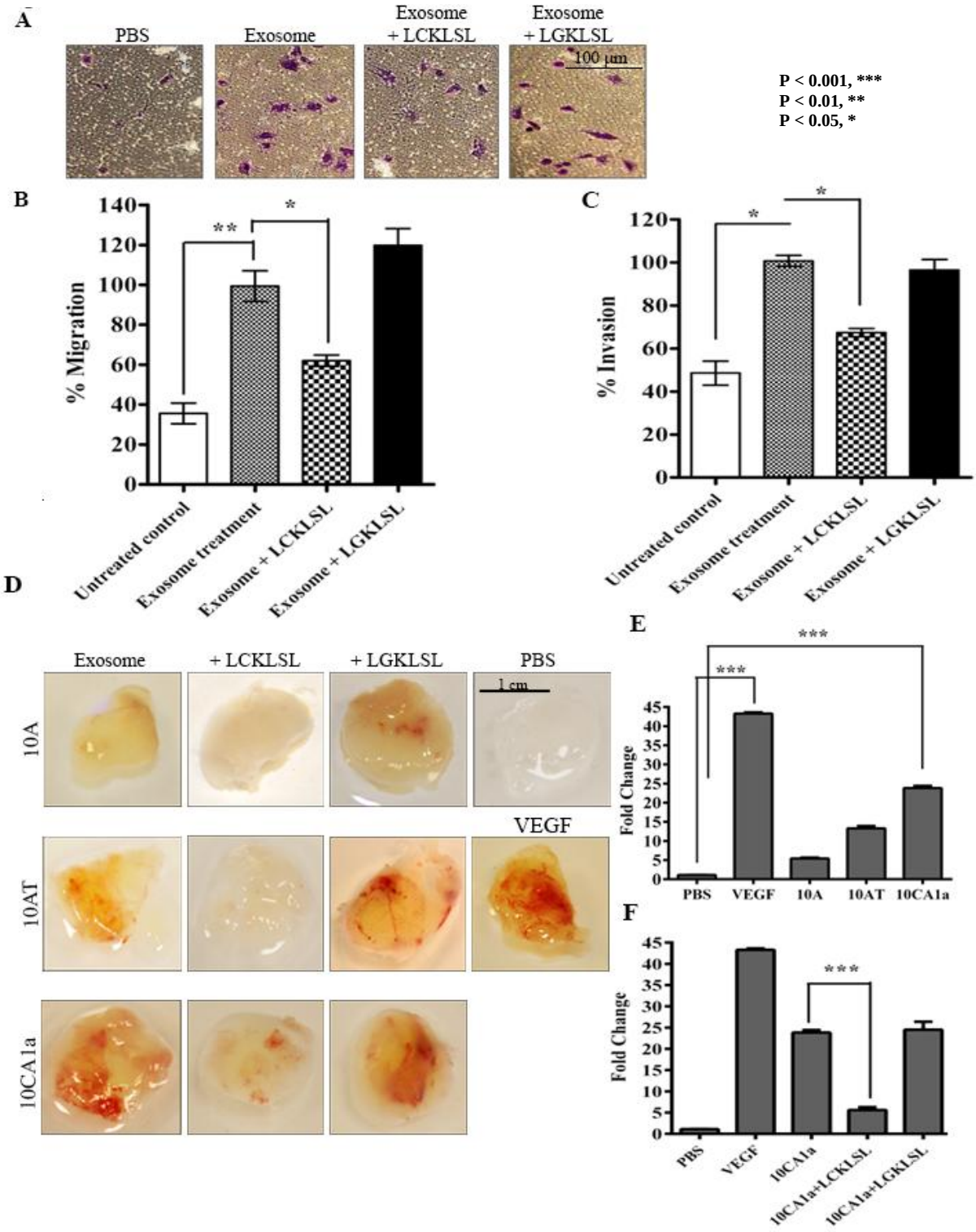
A
PBS
Exosome
Exosome + LCKLSL
Exosome + LGKLSL
100 µm
P < 0.001, ***
P < 0.01, **
P < 0.05, *
B
% Migration
Untreated control
Exosome treatment
Exosome + LCKLSL
Exosome + LGKLSL
C
% Invasion
D
Exosome
+ LCKLSL
+ LGKLSL
PBS
1 cm
10A
10AT
10CA1a
VEGF
E
Fold Change
PBS
VEGF
10A
10AT
10CA1a
F
Fold Change
PBS
VEGF
10CA1a
10CA1a+LCKLSL
10CA1a+LGKLSL

Figure 3: Exo-AnxA2 promotes angiogenesis (A-F). A) Images of HUVEC cell invasion assay treated in the following order (L to R) PBS, MCF10CA1a Exosomes, MCF10CA1a exosomes + LCKLSL peptide (5 µM) and MCF10CA1a exosomes + LGKLSL peptide (5 µM) (n=3). Quantification of migration (B) and invasion (C) of HUVEC cells after exosome treatment; Number of migrating or invading cells with MCF10CA1a exosome treatment is shown as 100% and percent change with different treatments is shown. Scale bar 100 µm. Migration assay was performed without matrigel and the number of cells migrating through the membrane was calculated. Invasion assay was performed in presence of matrigel and the number of cells invading through the matrigel was calculated.

D) Comparison of effects of exosomal treatment with LCKLSL or LGKLSL peptide in Matrigel plug assay. PBS (negative control), VEGF (100 ng/ml, positive control) and 100 µg of exosomal proteins were used. Representative images of matrigel plugs shown. Scale bar 1 cm. Hemoglobin estimation of homogenized matrigel plugs by Drabkin's method (E-F). Fold change to PBS is shown. Peptide concentration: 5 µM (n=3). Exosomes from MCF10A, MCF10AT and MCF10CA1a cells are designated as 10A, 10AT and 10CA1a respectively.

Fig. 4

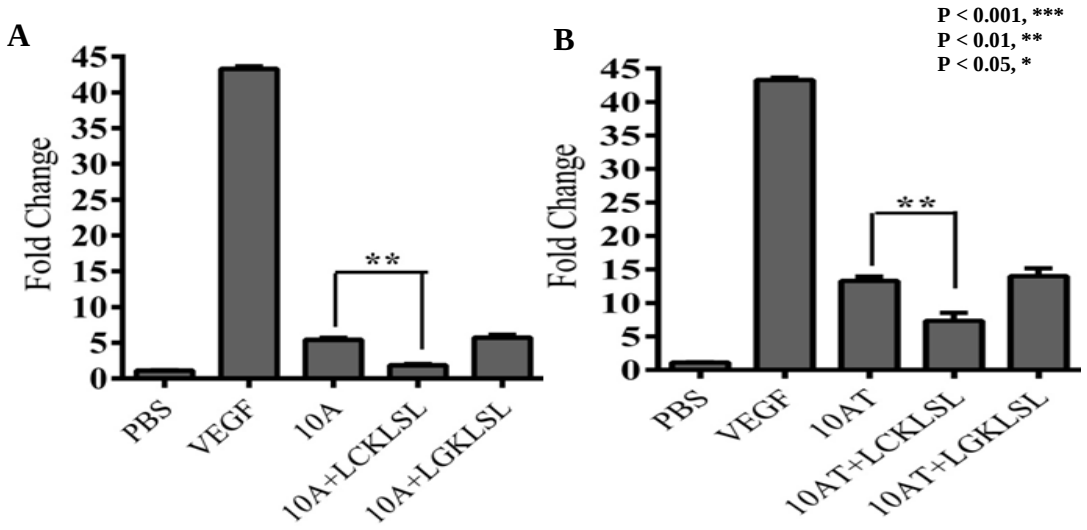

Figure 4: Quantification of matrigel hemoglobin content with MCF10A (A) and MCF10AT (B) exosomes in presence of LCKLSL or LGKLSL.

Fig. 5

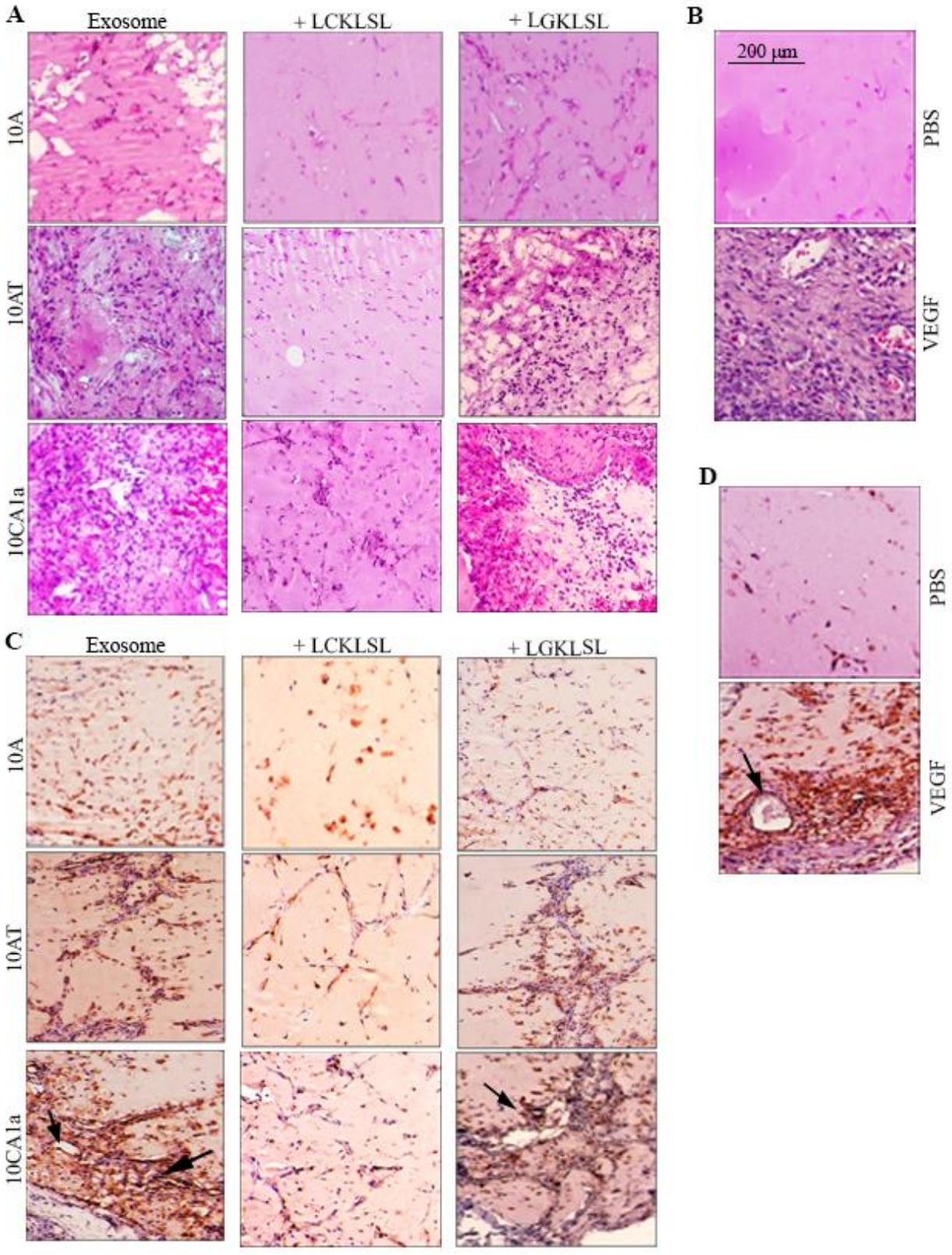
A
Exosome
+ LCKLSL
+ LGKLSL
10A
10AT
10CA1a
B
200 μm
PBS
VEGF
C
Exosome
+ LCKLSL
+ LGKLSL
10A
10AT
10CA1a
D
PBS
VEGF

Figure 5: Histological analysis of matrigel plugs A) H & E staining of MCF10A, MCF10AT and MCF10CA1a matrigel plug sections. B) H & E staining of PBS and VEGF matrigel plug sections. C) DAB staining against CD31 of MCF10A, MCF10AT and MCF10CA1a matrigel plug sections and D) PBS and VEGF matrigel plug sections (n=2). Arrowhead marks the capillaries. Scale bar 200 µm. Exosomes from MCF10A, MCF10AT and MCF10CA1a cells are designated as 10A, 10AT and 10CA1a respectively.

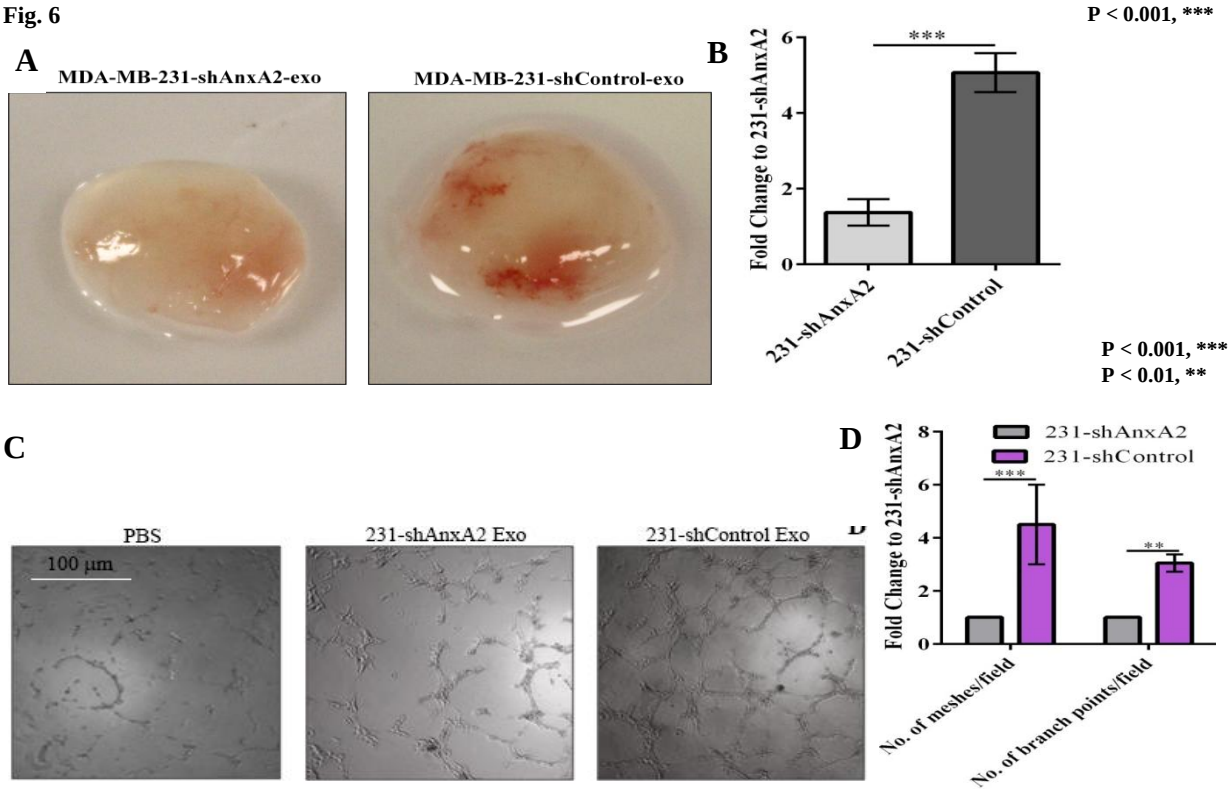

Figure 6: *In vivo* matrigel plug assay with MDA-MB-231-shAnxA2-Exo or MDA-MB-231-shControl-Exo (A) and hemoglobin content quantification (B). *In vitro* endothelial tube formation assay with MDA-MB-231-shAnxA2-Exo or MDA-MB-231-shControl-Exo (C) and its quantification (D) are shown. Scale bar 100 µm

Discussion:

Our study explores an important function of an abundant exosomal protein – AnxA2 in angiogenesis. In the previous chapter we saw via Western blot and AFM analysis of the exosomes in MCF10A progression model that exo-AnxA2 levels positively correlate with the aggressiveness of breast cancer. Upon exploring the role of exo-AnxA2 in angiogenesis we discovered that exo-AnxA2 can act as an important mediator of angiogenesis, as evident from our *in vitro* and *in vivo* angiogenesis experiments (Figs. 1 - 6). We found that exo-AnxA2 promotes network formation in *in vitro* endothelial tube formation assay as well as significant blood vessel formation in the *in vivo* matrigel plug assay. To confirm that the angiogenic effect is specifically due to exo-AnxA2 we used LCKLSL inhibitory peptide as well as performed shRNA mediated knock down of AnxA2. Angiogenesis is a sequential process that occurs in a series of three steps: initiation, proliferation-invasion and maturation (Kumar et al. 1998). In the first step, angiogenic inducers stimulate vascular cell proliferation and invasion. The activated endothelium secretes ECM-degrading proteases that permit the dissolution of the endothelial cell basement membrane, thereby promoting endothelial cell invasion and migration and finally stabilization of the new vessels. Consistent with our angiogenesis experimental findings, we discovered that exo-AnxA2 treatment increases migration and invasion of the endothelial cells (Fig. 3 A-C), further supporting our hypothesis.

We believe that exo-AnxA2 plays an important role in the tumor microenvironment by inducing angiogenesis via promoting migration, invasion of endothelial cells as well as increased plasmin generation, thereby aiding in primary tumor progression.

Reference:

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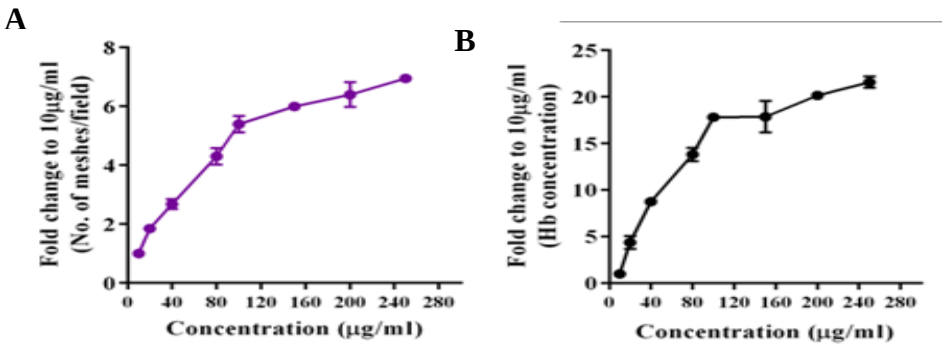
Supplemental Data:

Supplemental Figure S1:



Supplemental Figure 1: Dose response curves for determining the optimal exosomal concentrations for functional assays: Quantification of number of meshes per field from in vitro endothelial tube formation assay (A) and Hemoglobin estimation (B).

Exosomal Concentration: For all our functional studies, we chose 100µg/ml as the minimal effective exosomal protein concentration based on our preliminary dose response studies. We performed two dose response studies with different exosomal protein concentrations – *in vitro* endothelial tube formation assay and matrigel plug assay. Quantification of the number of meshes per field from endothelial tube formation assay (Supplementary Fig. S1 A) and (Supplementary Fig. S1 B) hemoglobin estimation from matrigel plug assay with different exosome concentrations are shown.

Processing of exosomes after peptide incubation:

For all the assays with LCKLSL or LGKLSL peptides, exosomal pellets were resuspended in PBS and incubated overnight with peptide. Next, the exosomes were re-centrifuged at 100,000g for 70 mins to remove unbound peptides and the exosomal pellet was again suspended in PBS for use in functional assays.

CHAPTER: IV. ROLE OF EXOSOMAL ANNEXIN A2 IN BREAST CANCER METASTASIS

Introduction:

The premetastatic niche is a specialized microenvironment that forms at the sites of future metastases and promotes the survival and outgrowth of disseminated tumor cells. According to recent reports systemic factors from the primary tumor might be involved in premetastatic niche formation. Accumulating evidence suggests that exosomes are major players in this process and act as mediators of metastasis (Peinado et al., 2012). Multiple cell types contribute to the formation of metastatic environments including fibroblasts, bone marrow progenitor cells, endothelial cells contribute to and probably utilize exosomes as a major tool for the horizontal transfer of proteins and nucleic acids. (Peinado et al., 2012) However, the underlying molecular mechanisms still need to be explored.

Recently Peinado *et al*. showed that the protein concentration in isolated exosomes from the plasma of patients with melanoma was significantly higher in the exosomes from patients with stage 4 disease (metastatic melanoma) than from patients with earlier stages of melanoma. In order to explore the role of exosomes in metastatic process, Peinado et al. showed that treatment with B16-F10 melanoma exosomes in animals leads to the up-regulation of many genes associated with premetastatic niche formation compared to controls. Furthermore, they showed that intravenous injection of B16-F10 melanoma exosomes prior to challenge with B16-F10 cells led to significant increase in the metastatic tumor burden when compared to control-treated mice (Peinado et al., 2012). In their melanoma study Peinado et al. showed that tumour-derived exosomes promote can metastasis mainly by conditioning both the bone marrow and the premetastatic niche. Their study led to the conclusion that exosomes or rather specific exosomal proteins could potentially be used to identify patients who are more likely to develop metastatic

disease. Furthermore, study of exosomes and their role in organ specific metastasis can lead to the identification of new targets for antimetastatic therapy.

In the present study we explored the role of breast cancer exosomes, especially exo-AnxA2 and how it creates a pre-metastatic niche and promotes organ specific breast cancer metastasis. By using an animal model we showed that regular priming with breast cancer exosomes creates a microenvironment conducive of metastasis. Furthermore, we showed that exo-AnxA2 plays an important role in this process and down-regulation of AnxA2 leads to significantly lesser brain and lung metastasis.

Materials and Methods:

Cell Lines: For the metastasis studies, MDA-MB-231 (ATCC) and its organ specific metastatic variants were used: MDA-MB-831 (brain metastatic), and MDA-MB-4175 (lung metastatic). (MDA-MB-831 and MDA-MB-4175 were gifted by Dr. Joan Massagué, MSKCC and shipped by Antibody and Bioresource Core facility). The cells lines were authenticated by performing STR analysis with the Promega PowerPlex® Fusion V1.0. All the cells tested negative for mycoplasma infection when tested with MycoAlert plus from Lonza. The cell lines were not re authenticated by the authors.

shRNA mediated down-regulation of AnxA2: To downregulate exo-AnxA2, shRNA against AnxA2 (GIPZ Lentiviral Human ANXA2 and shRNA, GE Dharmacon) was used. Positive clones were selected through puromycin antibiotic selection. Exosomes isolated from shAnxA2 and shControl cells will be designated as shAnxA2-Exo and shControl-Exo respectively. Exosomes from MDA-MB-231, MDA-MB-831 and MDA-MB-4175 cells will be designated as MDA-MB-231-Exo, MDA-MB-831-Exo and MDA-MB-4175-Exo respectively; exosomes from MDA-MB-231/831/4175-shControl and MDA-MB-231/831/4175-shAnxA2 cells will be

designated as MDA-MB-231/831/4175-shControl-Exo and MDA-MB-231/831/4175-shAnxA2-Exo respectively.

Animals: The animals were procured under IACUC approved protocols and all the studies and all the experiments were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) protocol for animal handling. 4-6 weeks old female athymic nude mice (Harlan Laboratories) were used for all the animal studies.

Priming of animals with exosomes: To study the role of exosomal AnxA2 in metastasis, the animals were injected with either PBS, exosomes from breast cancer cells stably transfected with sh-Control or with sh-AnxA2. Priming was performed via injection of 100 µg of exosomal proteins in 100 µl PBS via lateral tail vein, every three days for one month.

Organ tropism study: In order to study the role of breast cancer exosomes in organ tropism we injected female athymic nude mice with 100 µg of fluorescently labeled exosomes via tail vein. MDA-MB-231, MDA-MB-831 and MDA-MB-4175 exosomes were used for this study. After 24 hrs the mice were sacrificed and the lungs were harvested, fixed, and sectioned. Sections of the lungs were imaged under the Confocal microscope. Number of fluorescently tagged exosomes per field was counted.

Metastasis models: To check the role of exosomal AnxA2 in promoting breast cancer metastasis, two different models were used: intravenous (lateral tail vein) lung metastasis and intra cardiac metastasis (ic) model. Highly aggressive breast cancer cell line MDA-MB-231 and its organ specific metastatic variants MDA-MB-231-831 (which preferentially metastasizes to the brain), and MDA-MB-231-4175 (which preferentially metastasizes to the lung) cell lines were used for the metastasis study (Zhang et al., 2009). At the end of exosome priming, the primed animals were challenged with the respective luciferase positive breast cancer cells and then the extent of

metastasis to different organs was studied via bioluminescence analysis over time. Difference in the extent of metastasis to different organs between the control and treatment groups indicated the contribution of exosomal AnxA2 in breast cancer metastasis. At the end of the study, the animals were sacrificed and the organs were harvested and analyzed further via Hematoxylin and Eosin (H and E) staining as well as DAB immunostaining against human Vimentin (NCL-L-VIM-V9, Leica Biosystems) to stain the metastasized human breast cancer cells.

Intravenous (tail vein) injection of luciferase positive breast cancer cells: Intravenous injection of MDA-MB-231 cells was performed as published previously. (Liang et al., 2005) and (Adiseshaiah et al., 2014). Briefly, 2 x 10^6 MDA-MB-231-luciferase cells were resuspended in 50µl cold PBS and injected into the lateral tail vein of mice and bioluminescence imaging (BLI) was performed once every week till sacrifice.

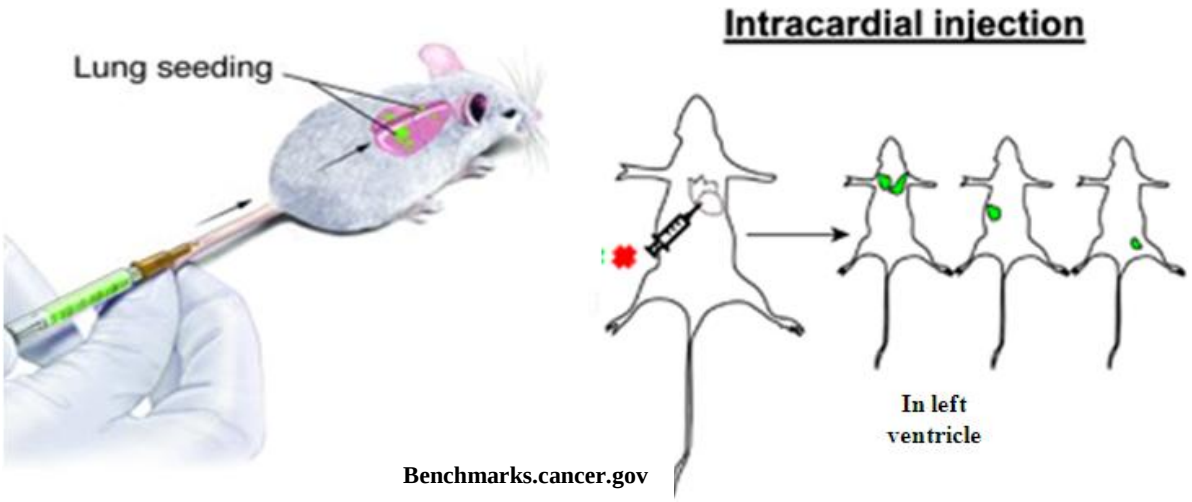


Figure 1. Models of metastasis: tail vein lung metastasis model (left panel) and intracardiac metastasis model (right panel)

Intracardiac inoculation of breast cancer cells: Female nude mice (age 6-8 weeks) were anesthetized by intramuscular injection of 120 mg/kg ketamine hydrochloride with 6 mg/kg body weight xylazine on the day of injections, and by exposure to 1–3% isoflurane on subsequent

imaging days. On day 0, anesthetized animals were injected with 1×10^5 luciferase positive MDA-MB-231/MDA-MB-831 or MDA-MB-4175 cells suspended in 100 µl sterile DPBS into the left ventricle of the heart. After the injection, anesthetized mice were placed in the IVIS™ Imaging System and imaged from both dorsal and ventral views approximately 10–15 mins after intraperitoneal injection of D-luciferin. Systemic bioluminescence distribution throughout the animal day 0 demonstrated successful (ic) injection. Only mice with evidence of a satisfactory injection were continued in the experiment. Assessment of subsequent metastasis was monitored *in vivo* once a week by imaging for up to 5 weeks. (Jenkins et al., 2005)

In vivo imaging of animals:

After the injection of luciferase positive breast cancer cells the metastasis was measured using non-invasive bioluminescence imaging, after intraperitoneal (IP) injection of 150 mg/kg of D-luciferin (Caliper Life Sciences Inc. MA, USA) using IVIS Lumina XR system (Caliper Life Sciences Inc. MA, USA). The animals were kept continuously under isoflurane during the whole imaging process. The change in photo flux over the time considered as an indicative of metastasis. Metastasis measured the very next day of inoculation was considered as the baseline values followed by once a week measurements. All the animals were imaged regularly till sacrificed.

Immunoprecipitation and Signaling: For the immunoprecipitation experiment, MDA-MB-231-shAnxA2 cells were treated with PBS/ 231-shAnxA2-exo or 231-shControl-exo (100 µg protein) and returned to the incubator for 6 hrs. After 6 hrs, the cells were washed with PBS and the membrane proteins were stripped via Versene wash (0.5 mM EDTA and PBS buffer). The EDTA eluates were centrifuged at 10,000g for 15 mins to remove any cell debris and immunoprecipitated with pro-cathepsin B antibody overnight and probed for AnxA2. For the

signaling experiments, MDA-MB-231 or HUVEC endothelial cells were treated with PBS/ 231-shAnxA2-exo or 231-shControl-exo (100 µg of exosomal protein) and returned to the incubator for 6 hrs. After 6 hrs the cells were washed with PBS and WCL was collected in NP40 lysis buffer and Western immunoblotting was performed with the designated antibodies.

Antibodies and reagents: The following antibodies were used: anti-AnxA2 (BD Biosciences), anti-GAPDH, anti-CD81 (Santa Cruz Biotechnologies), phospho p38 (Thr180/ Tyr182), p38, phospho NFκB p65 (S536), NFκB p65, phosphoSTAT-3 (Y705), STAT-3, MMP-9, Human Vimentin antibody (NCL-L-VIM-V9, Leica Biosystems), VEGFR1 (Abcam), protein AG beads for immunoprecipitation (Santa Cruz Biotechnologies), Versene (Gibco), India ink dye (American Master Tech.), PKH26 (Sigma Aldrich), Matrigel and matrigel-HC (BD Biosciences), D-luciferin (Caliper Life Sciences).

Exosome isolation: Exosomes were isolated by ultracentrifugation (Lasser et al., 2012) and were labeled with PKH26 (Sigma Aldrich) as published previously (Franzen et al., 2014).

Statistical Analysis:

Results are expressed as arithmetic means ± SEM if not otherwise indicated. Values of $P \leq 0.05$ were considered statistically significant, as determined by the unpaired Mann–Whitney test, the two-tailed unpaired Student *t* test, or the Wilcoxon signed rank test where appropriate. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ for all the figures. Statistical software GraphPad Prism was used for all statistical analysis. NCSS Power analysis software was used for animal studies.

Results:

Breast cancer exosomes have organ tropism: We found that intravenous (tail vein) exosome injection leads to organ tropism of the breast cancer exosomes since fluorescently tagged MDA-MB-4175 lung metastatic exosomes showed ~5.5fold more lung localization than MDA-MB-231

or MDA-MB-831 exosomes (Fig. 2). This indicates a possible mechanistic cross-talk between the exosomes and the homing organ leading to organ tropism.

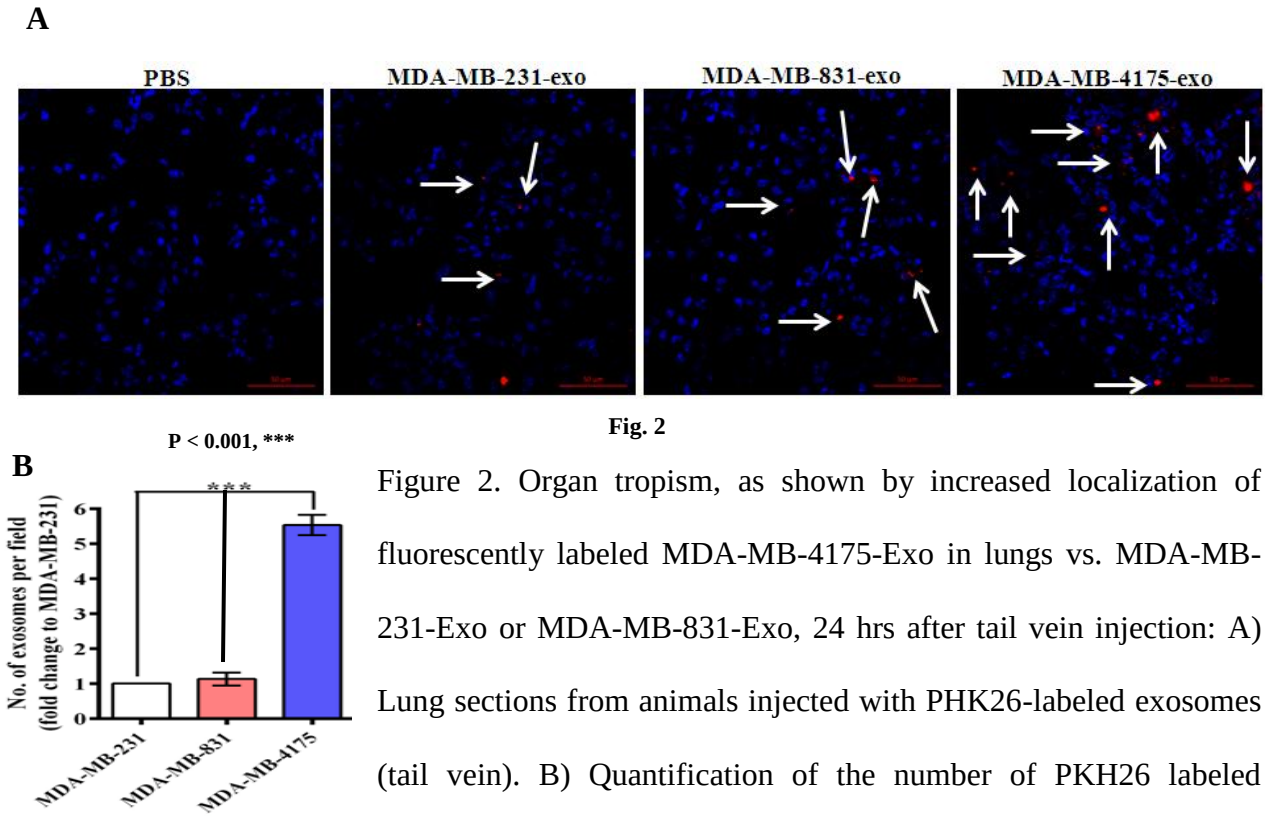


Fig. 2

Figure 2. Organ tropism, as shown by increased localization of fluorescently labeled MDA-MB-4175-Exo in lungs vs. MDA-MB-231-Exo or MDA-MB-831-Exo, 24 hrs after tail vein injection: A) Lung sections from animals injected with PHK26-labeled exosomes (tail vein). B) Quantification of the number of PKH26 labeled exosomes present per field (n=2). All the confocal images were taken with Zeiss LSM 510 confocal microscope at identical magnification and exposure and analyzed by LSM ZEN software.

Exo-AnxA2 promotes breast cancer metastasis to lungs: MDA-MB-231 and its organ specific metastatic variants MDA-MB-831 (brain metastatic) and MDA-MB-4175 (lung metastatic) cells were used for the breast cancer metastasis studies since they are very well characterized and are established models to study organ specific breast cancer metastasis (Zhang et al., 2009). First we tested the lung metastasis model by lateral tail vein injection. Challenge with 2×10^6 luciferase positive MDA-MB-231-luc cells (tail vein) after one month of exosome priming showed increased lung metastasis in MDA-MB-231-shControl-Exo primed animals than MDA-MB-231-

shAnxA2-Exo primed (~2fold) or PBS treated animals (~6fold) via bioluminescence imaging (BLI) (Fig. 3 C and 3 H). Quantification of the number of metastatic lung nodules showed priming with MDA-MB-231-shControl-Exo resulted in ~12fold and ~2.5fold increase in the number of nodules than PBS primed and MDA-MB-231-shAnxA2-Exo primed animals respectively (Fig. 3 E-F). We confirmed our results with H & E and human vimentin immunostaining of the lung sections which showed significant metastasis in MDA-MB-231-shControl-Exo primed animals than MDA-MB-231-shAnxA2-Exo primed animals (Fig. 3 J). Both H & E as well as vimentin staining showed large metastatic lung nodules in MDA-MB-231-shControl-Exo primed animals, whereas in MDA-MB-231-shAnxA2-Exo primed animals metastatic nodules were comparatively much smaller.

We further studied the role of exo-AnxA2 in lung metastasis in a different metastatic model: (ic) injection with MDA-MB-4175 lung metastatic cells. Challenge with 1×10^5 luciferase positive MDA-MB-4175-luc cells after one month of exosome priming showed increased lung metastasis in MDA-MB-4175-shControl-Exo primed animals than MDA-MB-4175-shAnxA2-Exo primed animals (~1.8fold) or PBS treated animals (~2.4fold) via BLI (Fig. 3 D and 5 I). Quantification of the number of metastatic lung nodules showed priming with MDA-MB-4175-shControl-Exo resulted in ~8fold and ~2fold increase in the number of nodules when compared to PBS and MDA-MB-4175-shAnxA2-Exo primed animals respectively (Fig. 3 G), similar to our MDA-MB-231-luc results; further confirming the important role of exo-AnxA2 in lung metastasis.

Fig. 3

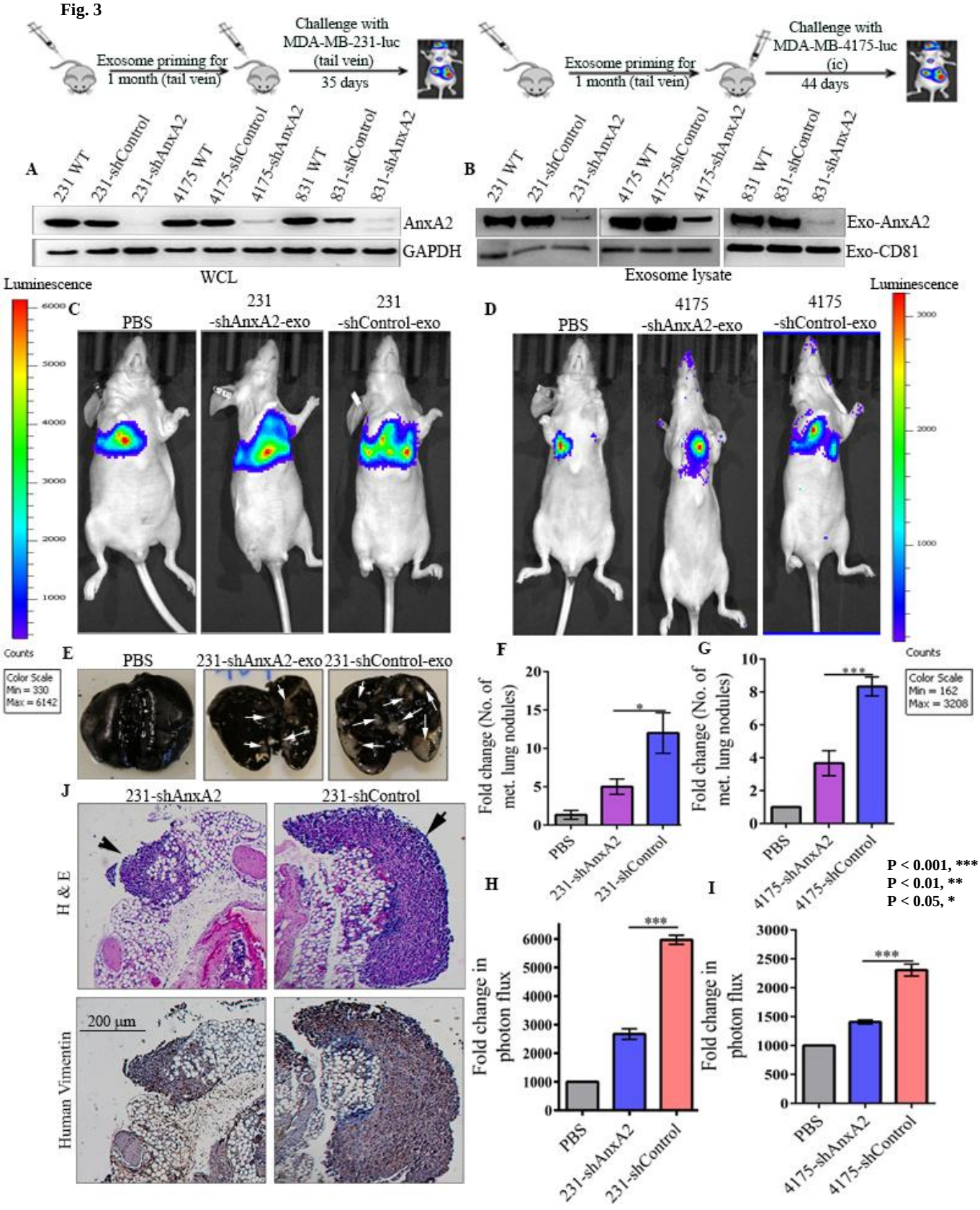

Exosome priming for 1 month (tail vein)
Challenge with MDA-MB-231-luc (tail vein) 35 days
Exosome priming for 1 month (tail vein)
Challenge with MDA-MB-4175-luc (ic) 44 days
A
231 WT
231-shControl
231-shAnxA2
4175 WT
4175-shControl
4175-shAnxA2
831 WT
831-shControl
831-shAnxA2
AnxA2
GAPDH
WCL
B
Exo-AnxA2
Exo-CD81
Exosome lysate
Luminescence
C
PBS
231 -shAnxA2-exo
231 -shControl-exo
D
4175 -shAnxA2-exo
4175 -shControl-exo
Counts
Color Scale Min = 330 Max = 6142
E
PBS
231-shAnxA2-exo
231-shControl-exo
Color Scale Min = 162 Max = 3208
F
Fold change (No. of met. lung nodules)
PBS
231-shAnxA2
231-shControl
G
4175-shAnxA2
4175-shControl
P < 0.001, ***
P < 0.01, **
P < 0.05, *
J
231-shAnxA2
231-shControl
H & E
Human Vimentin
200 µm
H
Fold change in photon flux
I

Figure 3: Exo-AnxA2 promotes breast cancer metastasis to lungs (A-I). A) Western blot analysis of WCL (A) and exosomal lysates (B) showing knockdown of AnxA2 (n=2) GAPDH and CD81 were used as loading controls for (A) and (B) respectively. C) Representative images of BLI of PBS or MDA-MB-231-shAnxA2-Exo or MDA-MB-231-shControl-Exo primed animals 35 days after challenge with MDA-MB-231-luc cells (lateral tail vein injection) showing difference in the extent of lung metastasis (n=8). D) Representative images of BLI of PBS or MDA-MB-4175-shAnxA2-Exo or MDA-MB-4175-shControl-Exo primed animals 44 days after challenge with MDA-MB-4175-luc cells (ic) showing difference in the extent of lung metastasis (n=8). E) India ink staining of the excised lungs from MDA-MB-231-luc cells injected animals showing the number of metastatic nodules. Quantification of the number of metastatic lung nodules with MDA-MB-231-luc (tail vein injection) treatment (F) and MDA-MB-4175-luc (ic) treatment (G). Fold change to PBS primed animals is shown. Quantification of the BLI in MDA-MB-231-luc challenged (H) and MDA-MB-4175-luc (I) challenged animals. Fold change in photon flux to PBS primed animals is shown. MDA-MB-4175-shAnxA2-exo or 4175-shAnxA2-exo, MDA-MB-231-shAnxA2-exo or 231-shAnxA2-exo, MDA-MB-4175-shControl-exo or 4175-shControl-exo and MDA-MB-231-shControl-exo or 231-shControl-exo terms have been used interchangeably. J) Representative images of H & E as well as DAB immunostaining against human vimentin of the lung sections showing the localized areas of lung metastasis (n=2). Arrow heads show the metastatic lung nodules. Scale bar 200 µm. All the H & E and DAB images were captured using a Nikon Phase contrast microscope attached with a Canon camera.

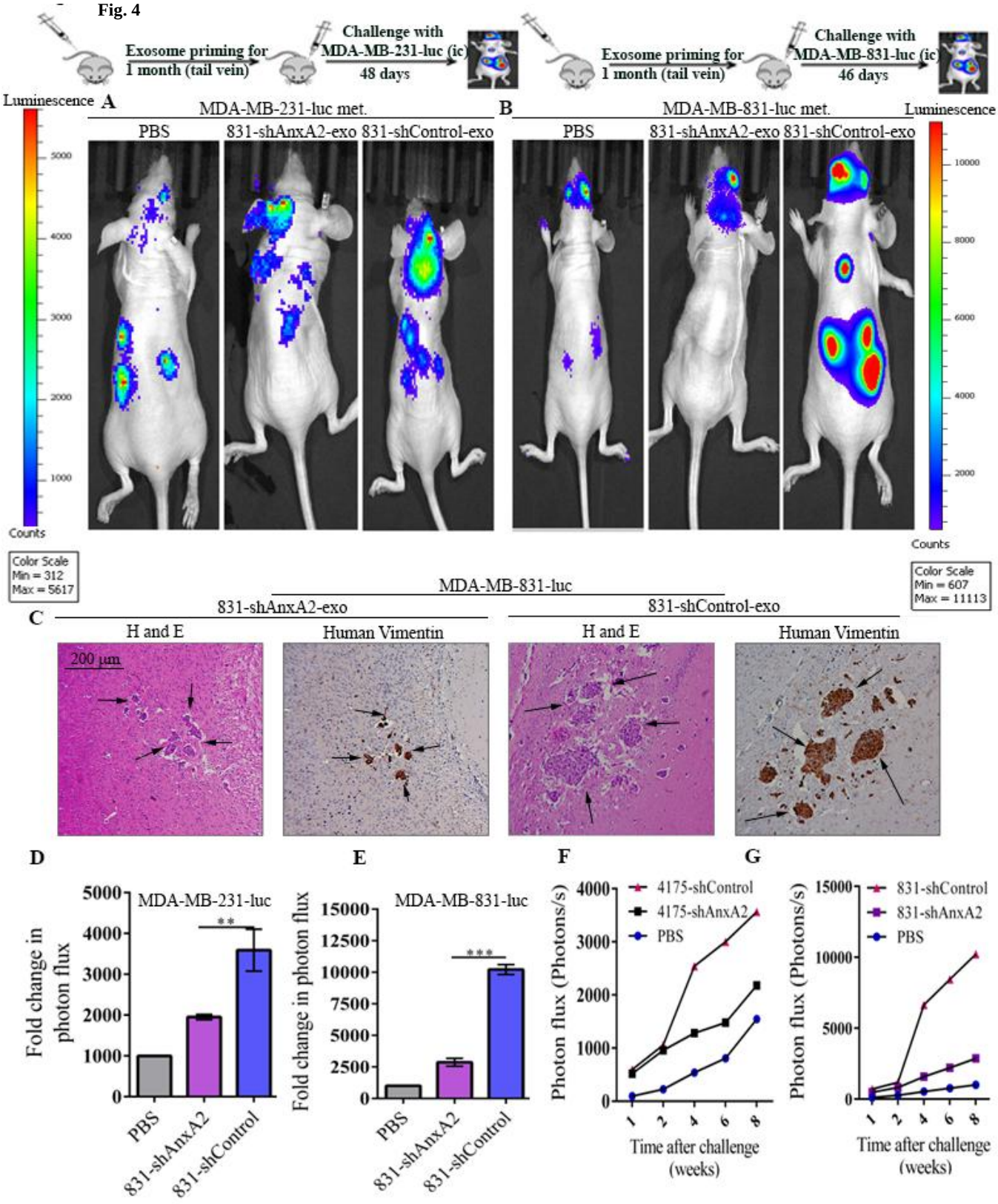
Exo-AnxA2 promotes breast cancer metastasis to brain: To study whether exo-AnxA2 promotes brain metastasis, we utilized two different experimental setups. First, we primed the animals with PBS or MDA-MB-831-shAnxA2-Exo or MDA-MB-831-shControl-Exo and then challenged with 1×10^5 MDA-MB-231-luc cells (ic). BLI of the animals revealed significant differences among the different treatment groups. Animals primed with MDA-MB-831-shControl-Exo showed increased brain metastasis when compared to MDA-MB-831-shAnxA2-Exo primed animals (~2fold) or PBS treated animals. (~3.8fold) (Fig. 4 A and 4 D).

Next we injected 1×10^5 MDA-MB-831-luc (ic) cells in PBS or MDA-MB-831-shAnxA2-Exo or MDA-MB-831-shControl-Exo primed animals. Similar to our MDA-MB-231-luc findings we saw significant differences among the different treatment groups but the differences were more pronounced than MDA-MB-231-luc challenged animals. Animals primed with MDA-MB-831-shControl-Exo showed significantly higher brain metastasis when compared to MDA-MB-831-shAnxA2-Exo primed animals (~4fold) or PBS treated animals (~10fold) (Fig. 4 B and 4 E). We confirmed our results with H & E and human vimentin immunostaining of the brain sections which showed significant macro metastasis in MDA-MB-831-shControl-Exo primed animals, whereas MDA-MB-831-shAnxA2-Exo primed animals showed micro metastasis. In both these brain metastatic studies, we found that exosome priming increases brain metastasis; especially in case of MDA-MB-231-luc injected animals, PBS priming showed whole body distribution of the cells, whereas in the exosome primed animals the cancer cells were more localized to the brain (Fig. 4 A).

Furthermore, in both the lung and brain metastatic models, we discovered that the respective exosome treatments led to a higher rate of organ specific metastasis (photon flux vs. time) in

MDA-MB-shControl-Exo primed animals (high bioluminescence starting from 2 weeks) than their shAnxA2-Exo counterparts suggesting that exo-AnxA2 priming creates a microenvironment that leads to faster and higher brain and lung metastases (Fig. 4 F-G). Increased brain specific metastasis also indicates that exosomes can cross the blood brain barrier as reported previously (Alvarez-Erviti et al., 2011) and form a premetastatic niche.

Fig. 4



P < 0.001, *
**P < 0.01, **
P < 0.05, *

Figure 4: Exo-AnxA2 promotes breast cancer metastasis to brain (A-G). A) Representative images of BLI of PBS or MDA-MB-831-shAnxA2-Exo or MDA-MB-831-shControl-Exo primed animals 48 days after challenge with MDA-MB-231-luc cells (ic) showing difference in the extent of brain metastasis (n=8). B) Representative images of BLI of PBS or MDA-MB-831-shAnxA2-Exo or MDA-MB-831-shControl-Exo primed animals 46 days after challenge with MDA-MB-831-luc cells (ic) showing difference in the extent of brain metastasis (n=8). C) Representative images of H & E as well as DAB immunostaining against human vimentin of the brain sections showing the localized areas of brain metastasis (n=2). Scale bar 200 µm. Quantification of the BLI in MDA-MB-231-luc challenged (D) and MDA-MB-831-luc (E) challenged animals. Fold change in photon flux to PBS primed animals is shown. Quantification of photon flux change over time: F) MDA-MB-4175-luc injected lung metastasis and G) MDA-MB-831-luc injected brain metastasis. MDA-MB-831-shAnxA2-exo or 831-shAnxA2-exo and MDA-MB-831-shControl-exo or 831-shControl-exo terms have been used interchangeably. All the H & E and DAB images were captured using a Nikon Phase contrast microscope attached with a Canon camera.

Exo-AnxA2 leads to activation of p38-NFκB and STAT 3 pathways and forms a pre-metastatic niche: Having found that exo-AnxA2 is an important mediator of organ specific metastasis, we studied the molecular pathways that might be affected by exo-AnxA2 priming. Upon screening of various pathways, we found that MDA-MB-231-sh-Control-Exo treatment activates STAT3, p38 and NFκB signaling cascades (~1.4fold, ~1.2fold and ~1.2fold respectively) in HUVEC endothelial cells than MDA-MB-231-sh-AnxA2-Exo treatment (Fig. 5 A-B). These signaling molecules are known to play significant roles in breast cancer tumorigenesis and metastasis

(deGraffenried et al., 2005). Next, we investigated the expression of these signaling molecules in brain and lungs from the different primed groups. Sections from MDA-MB-231-shControl-Exo primed animals showed higher phospho-STAT3 and phospho-p38-NFκB immunostaining in the lung and brain stroma than MDA-MB-231-shAnxA2-Exo primed animals (Fig. 5 C-D). MMP9 staining of lung and brain sections also showed similar results. Analysis of the lung staining revealed that the expression of phospho-STAT3 and phospho-p38-NFκB were mostly confined to the alveolar network and interstitial air sacs but not in bronchial epithelial cells.

Fig 5

P < 0.001, *
**P < 0.01, **
P < 0.05, *

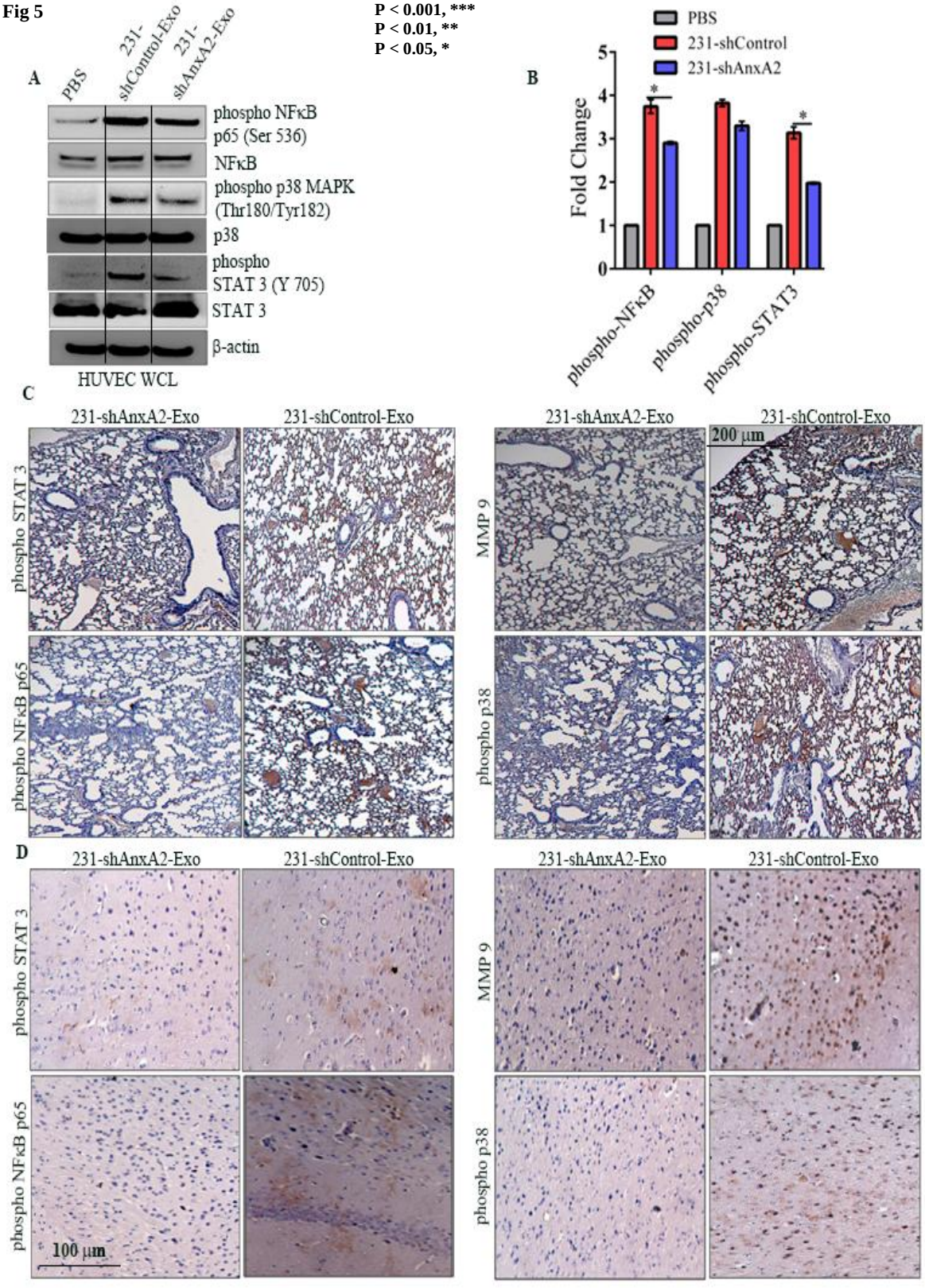


Figure 5: Exo-AnxA2 activates p38-NFκB and STAT3 signaling pathways. A) Western immunoblotting of HUVEC WCLs with the designated antibodies 4 hours after treatment with PBS or MDA-MB-231-shAnxA2-Exo or MDA-MB-231-shControl-Exo (n=2). B) Quantification of the blots normalized to β–actin. Fold change to PBS is shown.

Exo-AnxA2 creates a premetastatic niche promoting metastasis*:* Immunohistological analysis of lung (C) and brain (D) sections from MDA-MB-231-shAnxA2-Exo and MDA-MB-231-shControl-Exo primed animals. Representative images of DAB immunostaining for the respective proteins are shown. Scale bar 200 µm for lung sections and 100 µm for brain sections.

Breast cancer exosome priming leads to increased VEGFR1 expression in the primed organs:

During our metastasis studies we made another important observation; priming with MDA-MB-231-shControl-Exo showed increased levels of VEGFR1 in both lung and brain sections than PBS primed animals (Fig. 6) indicating that priming with cancer exosomes can up-regulate VEGFR1 expression.

Fig 6

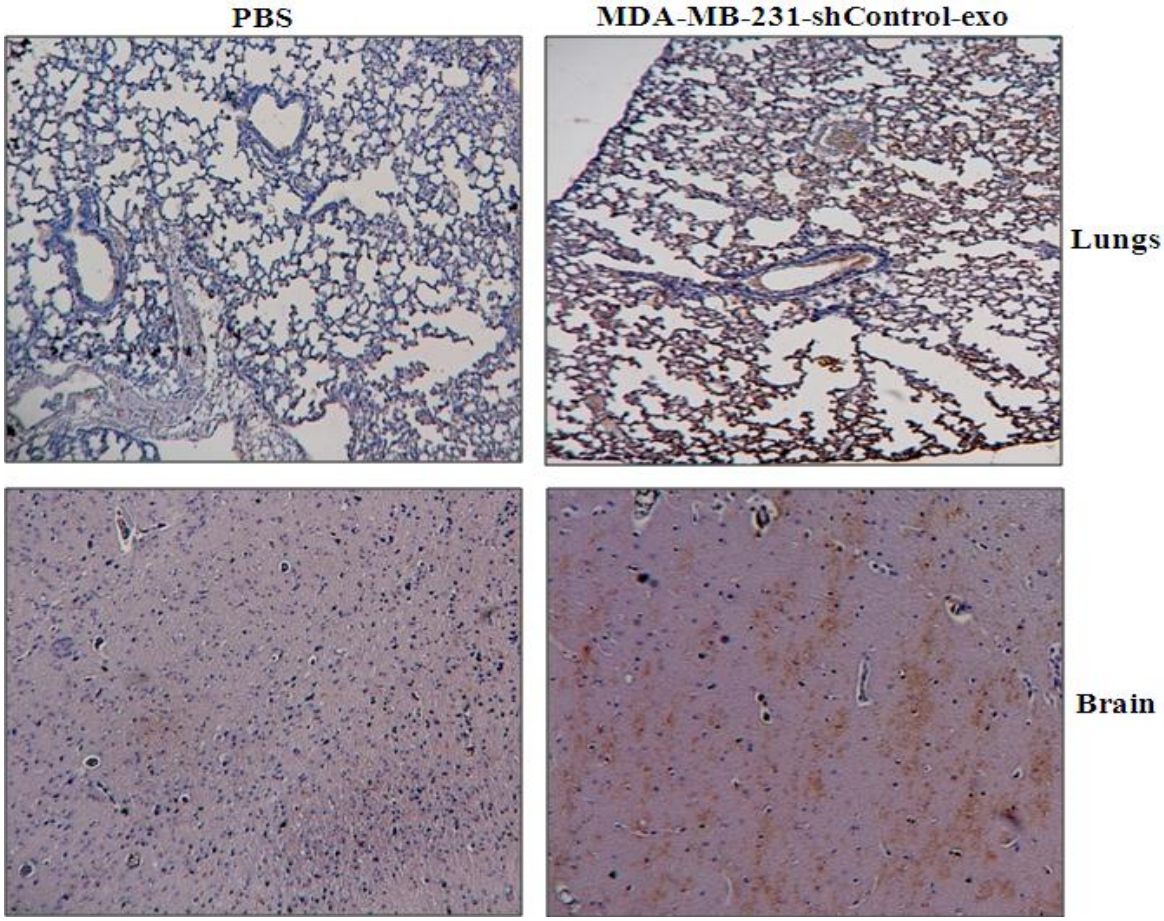


Figure 6: VEGFR1 DAB immunostaining in lung (upper panel) and brain (lower panel) sections from PBS or MDA-MB-231-shControl-Exo primed animals. Scale bar 200 µm for lung sections and 100 µm for brain sections.

Exosomal AnnexinA2 co-localizes with pro-cathepsin B or target cell surface: A preliminary analysis of the effect of exo-AnxA2 on MDA-MB-231-shAnxA2 breast cancer cells showed that once uptaken, exo-AnxA2 colocalizes with pro-cathepsinB on the cell surface (Fig. 7)

Fig 7

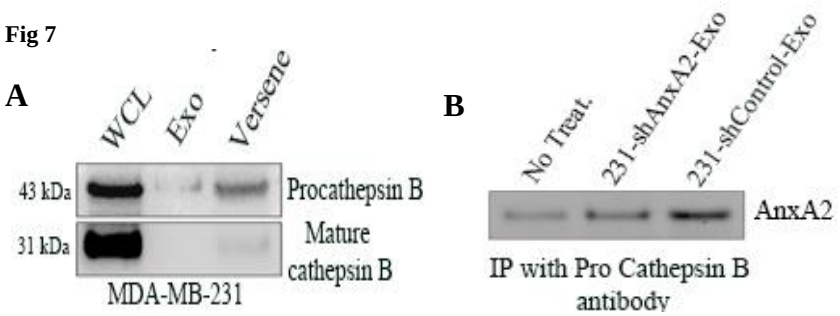


Figure 7: Exo-AnxA2 co-localizes with cathepsin B*:* A) Pro-cathepsin B and cathepsin B expression in WCL, membrane wash and exosomal lysate of MDA-MB-231-shAnxA2 cells. B) Membane versene washes from MDA-MB-231-shAnxA2 after treatment with PBS/ 231-shAnxA2-exo or 231-shControl-exo (100 μg exosomal proteins) for 6 hrs were immunoprecipitated with Cathepsin B antibody and probed for AnxA2 (n=2).

Discussion:

The premetastatic niche is a specialized microenvironment that forms at the sites of future metastases and promotes the survival and outgrowth of disseminated tumor cells. Evidence suggests that systemic factors (exosomes, microvesicles, etc.) from the primary tumor are involved in premetastatic niche formation, a specialized microenvironment that forms at the sites of future metastases and promotes the survival and outgrowth of disseminated tumor cells (Alderton et al., 2012). It has been shown previously that cancer exosomes can create a microenvironment conducive of promoting metastasis. Using an animal model, Peinado et al. showed for the first time that melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype (Peinado et al., 2012). Based on the concept that exosomes forms a premetastatic niche and promotes organ specific metastasis, we primed the animals with exosomes at regular intervals and then challenged the animals with cancer cells. We found that

priming of animals with exosomes leads to organ tropism as evident from the fact that MDA-MB-4175 lung metastatic exosomes shows much higher lung localization than MDA-MB-231 or MDA-MB-831 exosomes as early as 24 hours (Fig. 2). This is also supported by the data from our (tail vein) and (ic) metastasis models which show that priming with MDA-MB-4175 lung and MDA-MB-831-brain metastatic exosomes leads to increased lung (Fig. 3 C-I) and brain metastasis (Fig. 4 A-E) respectively compared to PBS treatment. We further discovered that downregulating exo-AnxA2 levels not only led to decreased lung and brain metastasis but also lesser rates of organ specific metastases. These data suggest an important role of exo-AnxA2 in breast cancer metastases. Upon screening the signaling pathways being affected by exo-AnxA2 treatment in primed animals, we found that exo-AnxA2 promotes metastasis by forming a pre-metastatic niche by activating STAT3, p38-NFκB and MMP9. We believe that activation of p38-NF-κB mediated proinflammatory cytokines and chemokines recruit tumor promoting macrophages since secreted AnxA2 has been shown to activate macrophage for IL-1, IL-6, and TNF-α secretion through TLR4/MyD88- and TRIF-dependent pathways (Swisher et al. 2007, Swisher et al. 2010). The cytokines from macrophages may promote tumor progression and metastasis, such as IL-6 for pancreatic cancer (Zhang et al. 2013) and hepatoma (Ohishi et al. 2014). However, detailed analysis of the underlying mechanisms needs to be performed.

Another interesting finding was the expression of VEGFR1 in the lungs and brain of the primed animals. Analysis of VEGFR1 in brain and lung sections showed that VEGFR1 expression was highly increased in cancer exosome primed animals than PBS primed animals. VEGFR1 has been previously linked with breast cancer metastasis (Duda et al. 2006, Psaila et al. 2006) and it is highly likely that VEGFR1 upregulation with exosome priming is an important mechanism leading to increased metastases.

There is also a possibility that apart from creating a premetastatic niche, exo-AnxA2 secreted by cancer cells can be involved in autocrine and paracrine signaling, acting on the cancer cells itself and promoting migration, invasion and metastasis. A preliminary analysis of the effect of exo-AnxA2 on MDA-MB-231-shAnxA2 breast cancer cells shows that once uptaken, exo-AnxA2 colocalizes with pro-cathepsinB on the cell surface (Fig. 7) most probably in a complex along with tPA, plasminogen and p11 as indicated earlier (Mai et al. 2000) and helps in ECM degradation, plasmin generation and metastatic dissemination (Vasiljeva et al. 2006) of the breast cancer cells, but further studies are needed to confirm this.

We believe that exo-AnxA2 is an important component of the breast cancer – microenvironment signaling and leads to activation of MMPs as well as creates a premetastatic niche leading to distant organ specific breast cancer metastasis.

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CHAPTER: V. ANALYSIS OF CLINICAL SERUM SAMPLES FOR EXOSOMAL ANXA2 LEVELS

ANALYSIS OF BREAST CANCER SERUM SAMPLES FOR EXOSOMAL ANNEXIN A2 EXPRESSION: A PILOT STUDY

Tumor exosomes manipulate the metastatic cascade through angiogenesis, signal transduction interference by growth factor/receptor transfer, stromal remodeling, pre-metastatic niche formation, chemoresistance and genetic intercellular exchange. Thus, exosomes offer new opportunities for biomarker analysis and may provide novel specific tools or targets for therapeutic intervention. For example, it is known that anti-cancer drug resistance usually results from selective pressure of chemotherapy, together with mutations or epigenetic changes that make cells refractory to treatment. Interestingly, gene expression associated with vesicle transport correlates with chemo sensitivity profiles. Furthermore, it has been observed that cancer cells can use exosomes as a drug efflux mechanism potentially involved in drug resistance. Currently, the presence of malignant tumors is clinically determined by biopsy procurement – which in itself may have detrimental effects. Thus alternative minimally invasive methods would be highly advantageous to the diagnosis and prognosis of breast cancer and the subsequent tailoring of targeted treatments for individuals. Especially, early detection of cancer is vital to improved overall survival rates. Thus exosomes can be an attractive area for biomarker discovery and targeted therapy mainly because exosome proteomes of different origins include a common set of membrane and cytosolic proteins, and specific subsets of proteins, likely correlated to cell-type associated functions. This is particularly interesting in relation to their involvement in human diseases (Hendrix et al., 2011). Having the knowledge of exosome composition can provide a better understanding of the tumor-microenvironment interaction, but can also identify new biomarkers in patients' fluids. Circulating exosomes have been identified as having potential diagnostic relevance in some cancer types including ovarian cancer, glioblastomas and lung cancer (Rabinowits et al., 2009, Skog et al., 2008). The prevalence and clinical relevance of exosomes in sera from breast cancer patients is yet to be elucidated. Exosomal proteins have

certain advantages with respect to their cellular counterparts when considered for biomarker status – (i) Exosomes are enriched in low abundance and membrane proteins that are usually underrepresented in conventional proteomic lists, such as cell lysates or unfractionated biological fluids (ii) exosomes have a conserved set of common proteins that are essential for vesicle biogenesis, structure and trafficking, and therefore they can be traced back to the cells from which they originate from, and (iii) exosomes contain proteins specific for the pathological conditions that are differentially expressed from their physiological counterparts. Potential biomarkers are likely to be found in the third group (paraphrased from Reinhardt et al., 2012). In this study we analyzed 50 breast cancer serum samples and 50 age matched control samples with no breast cancer diagnosis for whole serum AnxA2 as well as exo-AnxA2. ELISA analysis was performed and AnxA2 levels were correlated with presence or absence of breast cancer as well as with breast cancer subtypes.

Materials and Methods:

Collection of serum samples: 50 breast cancer serum samples and 50 age matched control archived serum samples were obtained. The samples were stored at -80 and were thawed on ice prior to use. All the archived serum samples were acquired under Institutional Review Board (IRB) approved protocols. The serum samples were blinded before analysis.

Exosome isolation from breast cancer serum samples: Exosomes from breast cancer serum samples were isolated by using total exosome isolation reagent (Life technologies, catalog: 4478360) according to the manufacturer's protocol. Briefly, the serum samples were thawed on ice and centrifuged at 2000 × g for 30 minutes to remove cells and debris. 100 µL of this clarified serum sample was mixed with 20 µL of the reagent and by vortexing or pipetting up and down until there was a homogenous solution. This mixture was incubated at 2°C to 8°C for 30

minutes. After incubation, the sample was centrifuged at 10,000 × g for 10 minutes at room temperature. The supernatant was discarded and the exosomal pellet was resuspended in 50 µL of 1X PBS and processed further.

Serum AnxA2 analysis by ELISA: AnxA2 levels in serum samples as well as serum exosomes were analyzed by ELISA kit (R&D systems) according to manufacturer's protocol. Briefly, 96 well microplate was coated with capture antibody overnight at 4 degrees, washed thrice and blocked with blocking buffer for 1-2 hrs at room temperature (RT). Next, the plates were incubated with the serum samples or serum exosomal samples diluted in IC diluents (5 fold dilutions) for 2 hours at RT. The plates were washed and coated with detection antibody for 2 hours at RT and washed again. The plates were incubated with Streptavidin-HRP for 20 mins at RT, washed and further incubated with TMB peroxidase substrate. The reaction was stopped using 2N H_2SO_4 and the optical density was read at 450 nm with wavelength correction at 540nm. Each samples/ standard was run in duplicates. (n=2)

Data analysis: Box plot analysis was used to plot the serum AnxA2 and serum exosome AnxA2 levels. Scatter plot analysis was used to analyze the correlation of serum AnxA2 and serum exosomal AnxA2 levels with control, human epidermal growth factor receptor 2 positive (HER2+) and Triple Negative Breast Cancer (TNBC) subtypes. All the analysis was performed using Graph Pad Prism software.

Results:

Exosomal AnxA2 levels are higher in breast cancer serum samples than control samples: A total of 50 breast cancer serum samples and 50 age matched samples without any cancer diagnosis were screened for total AnxA2 as well as exo-AnxA2 levels. ELISA analysis of total

serum AnxA2 revealed that AnxA2 levels are slightly higher compared to control samples (median increase by ~2 fold, Fig. 1A and 1B).

Interestingly, breast cancer exo-AnxA2 levels were found to be much higher when compared to control samples (median increase by ~4.5 fold, Fig. 1C and 1D) indicating that breast cancer serum samples have higher exo-AnxA2 levels than their normal counterparts.

Fig.1

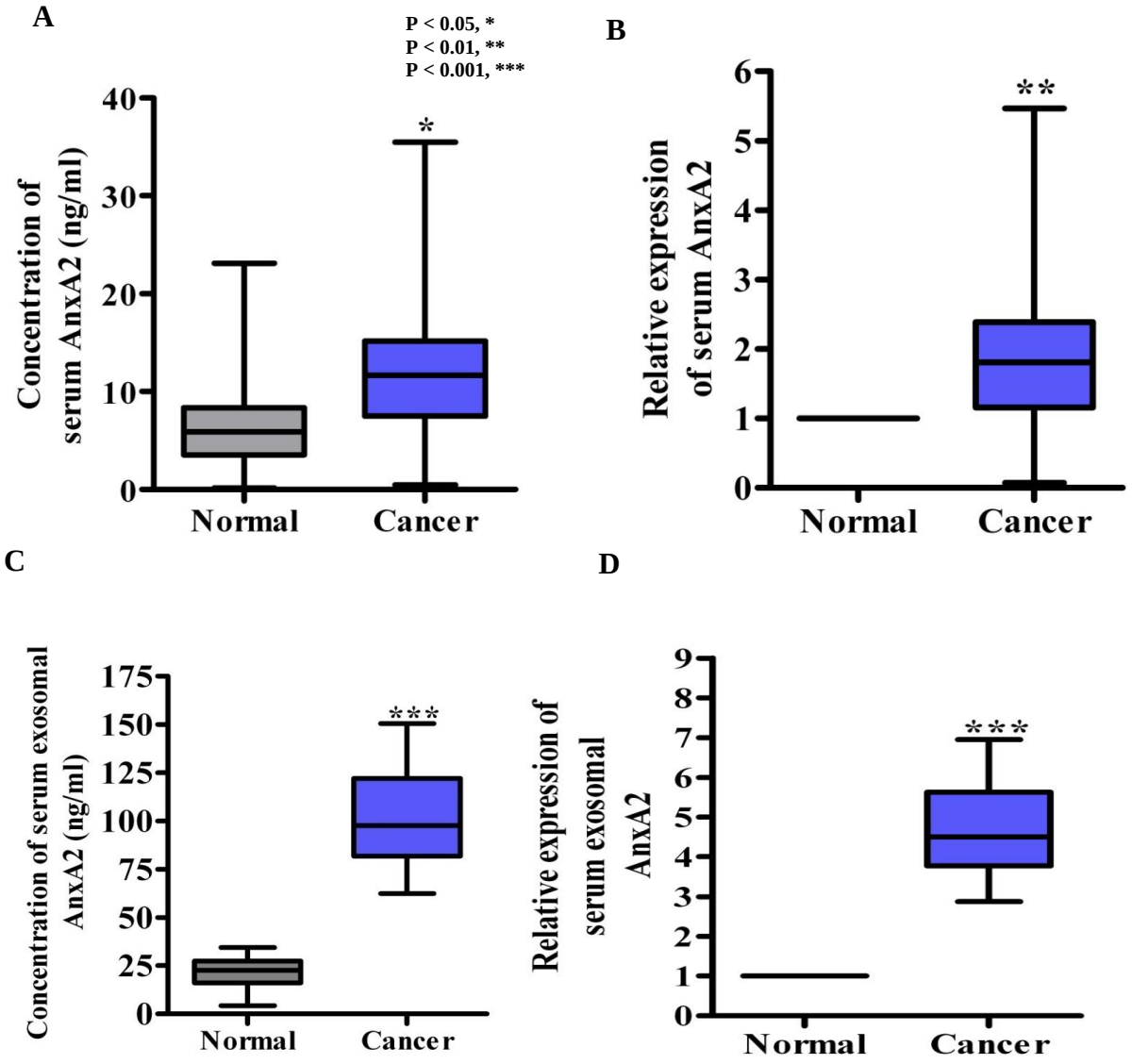

Figure 1: Exo-AnxA2 is highly expressed in breast cancer serum samples vs. control samples: Box plot depicting the total concentration (Fig. 1A) and relative expression (Fig. 1B) of serum AnxA2 as well as total concentration (Fig. 1C) and relative expression (Fig. 1D) of exo-AnxA2 in breast cancer and control serum samples. Protein estimation was performed by ELISA. (n=2)

Serum exosomal AnxA2 levels positively correlate with aggressive breast cancer subtypes: We further performed a scatter plot analysis plotting the correlation of exo-AnxA2 levels with the different breast cancer subtypes. We found that highly aggressive TNBC subtype has higher exo-AnxA2 levels (~1.6 fold) than HER2+ subtypes indicating that exo-AnxA2 might be overexpressed in aggressive breast cancer subtypes (Fig. 2A-B).

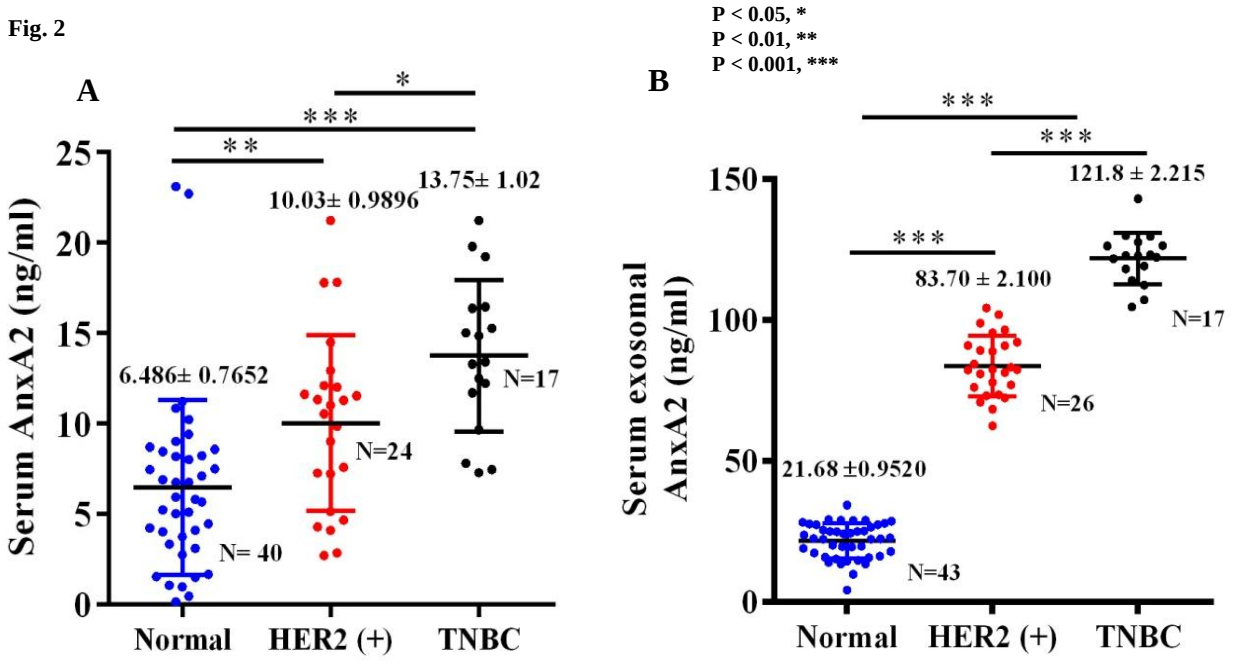

Figure 2: Scatter plot analysis of whole serum AnxA2 (Fig. 2A) and exo-AnxA2 levels (Fig. 2B) showing the correlation of AnxA2 levels with control samples, HER2+ and TNBC breast cancer samples.

Serum exosomal AnxA2 levels are higher in African-American subpopulation than Caucasian subpopulation: We further performed a scatter plot analysis plotting the correlation of exo-AnxA2 levels with the Caucasian and African-American subpopulations. We found that exo-AnxA2 levels are higher in African-American subpopulations than Caucasian subpopulations (~1.3fold higher) (Fig. 3 A). We further analyzed the serum exo-AnxA2 levels among Caucasian and African-American subpopulations in TNBC patient samples and found that similar to our previous findings, African-American subpopulation has higher levels of exo-AnxA2 than Caucasian subpopulation (~1.1fold higher) (Fig. 3 B), although the sample size for Caucasian TNBC samples very less to draw proper conclusions.

Fig. 3

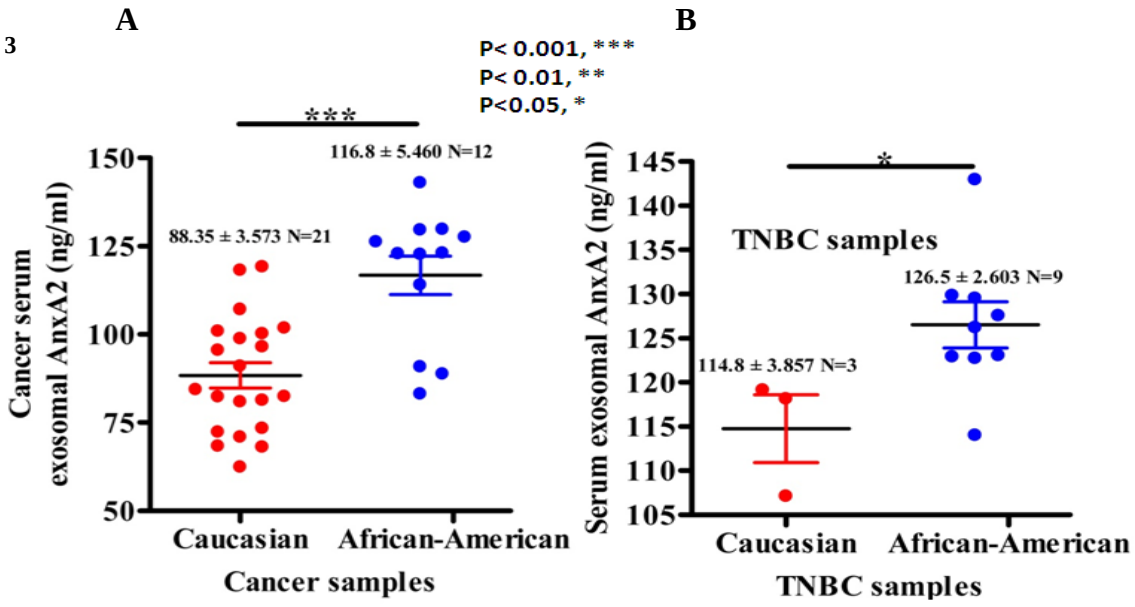

Figure 3: Scatter plot analysis of cancer serum exo-AnxA2 (A) and TNBC serum exo-AnxA2 (B) showing its distribution among Caucasian and African-American patients.

Inhibition of serum exosomal AnxA2 leads to decreased angiogenesis in an in vivo matrigel plug assay model: In our *in vitro* and *in vivo* angiogenesis studies with exosomes collected from cell culture supernatants we found that exo-AnxA2 is a promoter of angiogenesis. We further confirmed our findings in an in vivo matrigel plug assay experiment with exosomes collected from patient serum. We found that serum exo-AnxA2 is a potent inducer of angiogenesis as seen in the case of both normal and cancer serum matrigel plug assays (Fig. 4 A). However, the extent of angiogenesis was significantly higher in cancer serum exosomes matrigel plugs than normal serum exosomes matrigel plugs. When we incubated the serum exosomes with LCKLSL inhibitory peptides we saw significant decrease in degree of angiogenesis than incubation with LGKLSL control peptide (Fig. 4 A); confirming that serum exo-AnxA2 is a potent inducer of angiogenesis. We confirmed our findings by analyzing the extent of hemoglobin present in the matrigel plugs via Drabkin's method (Fig. 4 B and C).

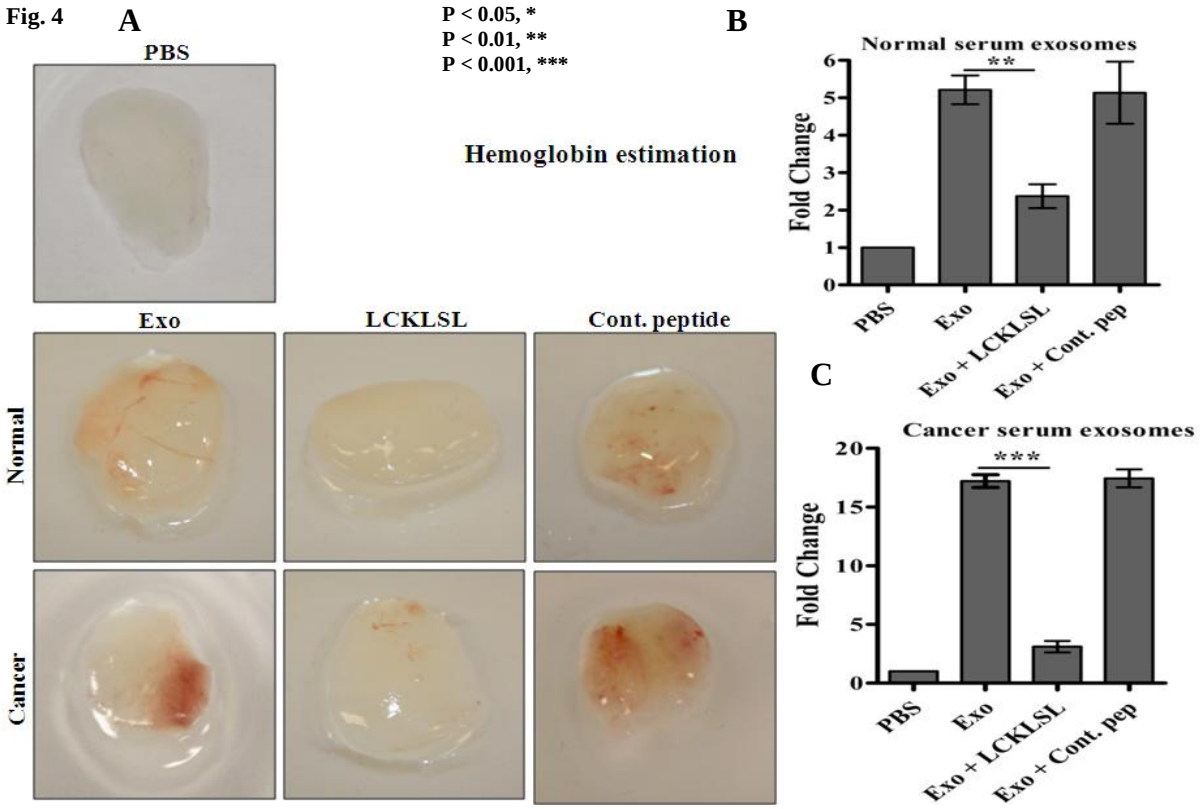

Figure 4: A) Matrigel plug assay with patient serum exosomes: Representative images of the matrigel plugs from matrigel plug assay with serum exosomes from normal and breast cancer patient samples along with incubation with LCKLSL inhibitory or LGKLSL control peptide. Quantification of hemoglobin estimation from matrigel plugs by Drabkin's method from normal serum (Fig. 4B) and cancer serum (Fig. 4C).

Discussion and future direction:

In this study we looked at the expression of whole serum AnxA2 as well as serum exo-AnxA2 in 50 breast cancer and 50 control serum samples. We found that exo-AnxA2 is highly overexpressed in cancer samples than control samples. Further correlation of exo-AnxA2 with HER2+ and TNBC breast cancer subtypes showed that exo-AnxA2 is higher in more aggressive TNBC subtypes than HER2+ subtypes indicating a possible role of exo-AnxA2 in breast cancer pathogenesis. We also found that exo-AnxA2 levels are higher in African-American subpopulation than Caucasian subpopulation. However, in order to validate the biomarker status of exo-AnxA2 in breast cancer metastasis, an in-depth analysis including a larger pool of serum samples and from different subtypes is needed. We plan to explore the correlation of serum exo-AnxA2 levels with other subtypes including Estrogen Receptor (ER) and Progesterone Receptor (PR) subtypes to have a better understanding of the functionality of exo-AnxA2 as well as discover any effect the presence or absence of these receptors might have on serum exo-AnxA2 expression. Also, we plan to correlate exo-AnxA2 levels with other ethnic subpopulations including Hispanic, Alaskan/ native American and Asian subpopulations. Furthermore, to better understand the correlation between serum exo-AnxA2 levels and metastasis, correlation of serum exo-AnxA2 in breast cancer patients with different organ specific metastases needs to be performed.

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CHAPTER VI: SUMMARY AND DISCUSSION

In this project, we looked at multiple facets of exo-AnxA2. We started with characterizing exo-AnxA2 in a breast cancer progression model. We discovered that exo-AnxA2 is high in malignant breast cancer cells than non-malignant and pre-malignant cells. Upon characterization we found that malignant breast cancer exosomes have higher surface expression of AnxA2 than its other counterparts indicating a possible role of exo-AnxA2 in breast cancer pathogenesis. Next we studied the role of exo-AnxA2 in angiogenesis. Molecular AnxA2 is an established inducer of angiogenesis but there are no reports regarding the function of exo-AnxA2. Using both *in vitro* and *in vivo* angiogenesis experiments we showed that exo-AnxA2 promotes angiogenesis which can be inhibited by using AnxA2 specific inhibitory hexapeptides that block the tPA binding site on AnxA2.

Upon studying the role of exo-AnxA2 in angiogenesis, we further explored its role in breast cancer metastasis. Using a well characterized organ specific breast cancer metastases model system we showed that regular treatment with breast cancer exosomes increases brain and lung metastases. We showed that downregulating exo-AnxA2 leads to lesser brain and lung metastases. We discovered that exo-AnxA2 leads to the activation of the p38-NFκB, STAT signaling cascades in the primed animals and creates a microenvironment conducive of promoting metastasis.

We further screened breast cancer and age matched control serum samples to detect the levels of serum exo-AnxA2. We found that exo-AnxA2 is ~4 fold overexpressed in breast cancer serum samples than control samples further supporting our hypothesis. Upon correlation of exo-AnxA2 levels with different breast cancer subtypes we discovered that exo-AnxA2 is overexpressed in highly aggressive TNBC breast cancer subtypes than HER2+ or control samples as well as African-American subpopulations than Caucasian subpopulations.

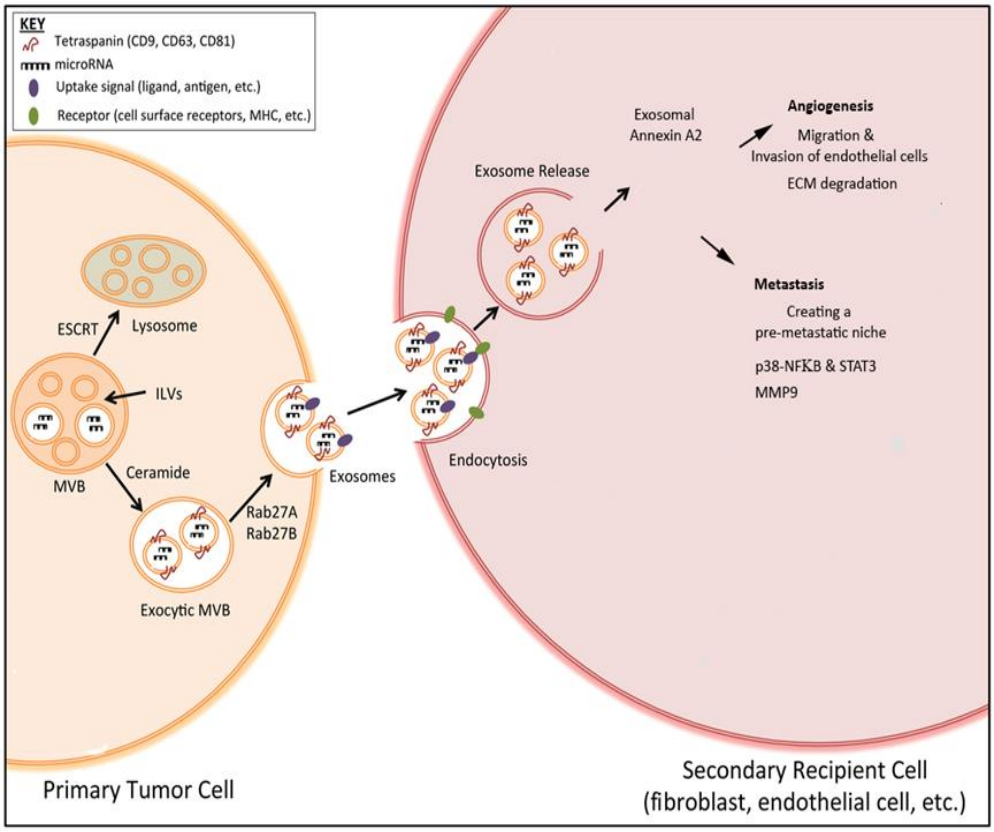

Figure 1: Proposed Model

We delineated the mechanism of action of breast cancer exosomal AnxA2. We found out that exosomal AnxA2 is taken up by recipient cells and promotes angiogenesis by ECM degradation as well as driving migration and invasion of endothelial cells. We also found that exosomal AnxA2 promotes organ specific breast cancer metastasis by creating a pre-metastatic niche probably by up-regulating p38-NFκB, STAT signaling cascades. Thus cancer exosomal AnxA2 is a major component of the tumor-microenvironment signaling (Fig. 1).

Exosomes are being reported as a rich source of tumor-specific proteins and thus are quickly emerging as prognostic and diagnostic biomarkers for cancer. Exosomes are known to contain specific protein and nucleic acid signatures that depend on the type of disease as well as the site of origin. Identified exosomal protein signatures can be used to determine tumor characteristics and help devise an appropriate treatment plan.

Recently, Duijvesz et al. (Duijvesz et al. 2013) compared exosomal proteins derived from non-cancerous prostate cells and prostate cancer cells to measure their presence and significance. They identified 4 candidate protein biomarkers, which were differentially expressed between the two exosome populations, for prostate cancer (PDCD6IP, FASN, XPO1 and ENO1). These results indicate that exosome profiling can emerge as a non-invasive and specific diagnostic tool of the future for prostate cancer.

Some recent studies have identified a number of exosomal protein biomarkers for various diseases. In 2008, Skog et al. indicated that exosomal epidermal growth factor receptor vIII (EGFRvIII) may provide diagnostic information for glioblastoma since they detected glioblastoma-specific EGFRvIII in serum exosomes isolated from 7 out of 25 glioblastoma patients (Skog et al. 2008). Later, Graner et al. reported high levels of EGFR, EGFRvIII and TGF-beta in serum exosomes from patients with brain tumors (Graner et al. 2009).

In 2009, Nilsson et al. identified well known prostate cancer biomarkers PCA-3 and TMPRSS2:ERG in exosomes isolated from the urine of prostate cancer patients (Nilsson et al. 2009). In 2012, Chen et al. identified 24 urinary exosomal proteins that were differentially expressed between patients with bladder cancer and hernia.

Apart from cancer, exosomal proteins have also been hailed as promising biomarkers in other diseases including acute kidney injury (Zhou et al. 2006), Alzheimer's disease (Rajendran et al. 2006 and Saman et al. 2012) and Parkinson's disease (Alvarez-Erviti et al. 2011).

In conclusion, exosomes are important mediators of intercellular communication and play roles in disease pathogenesis. Research on the biology, function, and potential application of exosomes has increased exponentially over the past decade. Perhaps the most important biomedical utility of exosomes is their potential application as biomarkers in clinical

diagnostics. Thus, study of the mechanisms of action of exosomal proteins as well as screening of exosomal protein signatures in various diseases is extremely important as it can lead to the identification of various non-invasive prognostic and diagnostic biomarkers.

References:

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