INVESTIGATING THE ROLE OF CONNEXIN 32 IN BREAST CANCER

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ABSTRACT

INVESTIGATING THE ROLE OF CONNEXIN 32 IN BREAST CANCER

Connexins (Cx) are primary components of gap junctions, selectively allowing molecules to be exchanged between adjacent cells. Along with their channel forming functions, connexins play variety of roles in different stages in tumorigenesis, both dependent and independent of gap junctions in connexin and cancer dependent manner. Cytoplasmic accumulation of Cx32 was shown in some breast cancers; and compared to the primary tumors Cx32 is further upregulated in metastasis. However, the complete picture for the role of Cx32 in breast cancer remains to be elusive. Through overexpressing Cx32, its functions in breast cancer cells were investigated in Hs578T and MCF7 breast cancer cells. Cx32 overexpression increased cellular proliferation with significant increase in S phase in Hs578T cells with no significant change on MCF7 cells. Cx32 overexpression did not induce hemichannel activity in neither cell; it reduced gap junctional functions in Hs578T cells. Cx32 in both cells localized in cytoplasm did not form intercellular plaques, and decreased Cx43 expression. Cx32 overexpression reduced the migration and invasion capacity in both cells and in Hs578T cells showed reduction of mesenchymal and increase of epithelial marker expressions. In conclusion, Cx32 increases proliferation and decreases communication in Hs578T cells while not affecting MCF7 cells. It decreases aggressiveness and metastatic potential for both cell lines. Due to changes in gap junctional functions, Cx32 might be acting in relation to GJIC in Hs578T cells and outside of it in MCF7 cells. All in all, presence of Cx32 made Hs578T cells act similar to endogenously Cx32 expressing MCF7 cells.

ÖZET

MEME KANSERİNDE CONNEXİN 32'NİN ROLÜNÜN ARAŞTIRILMASI

Connexinler (Cx) hücrelerarası oluklu bağlantıların temel parçaları olup, hücrelerarası iletişiminde, komşu hücreler arasında molekülleri seçici olarak geçirerek rol alırlar. Kanal oluşumunda rol almalarının dışında, tümör oluşumunun farklı evrelerinde, oluklu bağlantılarla veya ayrı olarak connexin ve kanser türüne bağlı olarak fonksiyon gösterirler. Sağlıklı insan meme dokusunda Cx26 ve Cx43 ifade ediliyor olmalarına rağmen, bu proteinlerin ifadeleri meme kanserinin başlangıcında azalmıştır. Bir diğer taraftan Cx32 sitoplazmik proteine meme kanseri dokularının bazılarında rastlanmış ve birincil kanser dokularına kıyasla ifadesi metastaz yapan dokularda daha da artmıştır. Lakin, Cx32'nin meme kanserinde oynadığı rol hala tam olarak bilinmemektedir. Cx32'nin meme kanserindeki fonksiyonu, ifadesi arttırılarak Hs578T ve MCF7 meme kanseri hücrelerinde araştırılmıştır. Cx32, Hs578T hücrelerinin çoğalmasını arttırmış, buna karşılık MCF7 hücrelerinde çoğalmayı etkilememiştir. Bununla birlikte hücre döngüsü analizlerinde, Cx32, Hs578T hücrelerinin S fazında bulunan hücre oranında anlamlı bir değişikliğe neden olmuş, MCF7 hücrelerinde bir değişikliğe yol açmamıştır. Cx32 ifade artışı iki hücrede de yarım kanal fonksiyonu yaratmazken, Hs578T hücrelerinin oluklu kanal fonksiyonunu azaltmıştır. Cx32 iki hücrede de sitoplazmada konuşlanırken, plak oluşturmamıştır. Cx32 varlığı Cx43 ifadesini azaltırken, bu azalma Hs578T hücresinde anlamlıdır. Cx32 ifade artışı hücre hareketi ve invazyonunu iki hücrede de azaltırken, Hs578T hücrelerinde mezenkimal genleri azaltıp, epitel genlerde artışa neden olmuştur. Sonuç olarak Cx32 varlığı Hs578T hücrelerinde çoğalmayı arttırıp, iletişimi azaltırken MCF7 hücrelerinde bir değişikliğe neden olmamıştır. İki hücrede de saldırganlık hareket ve invazyonla birlikte azalmıştır. Oluklu bağlantı değişimlerinden dolayı, Cx32 Hs578T'de bağlantı ile ilgili olarak çalışırken, MCF7'da ilgisiz çalışmaktadır. Genel olarak Cx32 varlığı, Hs578T hücrelerini, halihazırda Cx32 ifade eden MCF7 hücreleri gibi davranmaya itmiştir.

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LIST OF SYMBOLS and ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
μ	Mu
3’	Three prime
5’	Five prime
BrDU	Bromodeoxyuridine
C°	Degree Celsius
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Dioxylribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GFP	Green florescent protein
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
kDa	Kilo dalton
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
OD	Optical density
PBS	Phospate Buffer Saline
pH	Potential of Hydrogen
RNA	Ribonucleic acid
Rpm	rotation per minute
SDS	Sodium dodecyl sulfate
TE	Tris -EDTA buffer

CHAPTER 1

INTRODUCTION

1.1. Gap Junctions and Connexins

Cells in a multicellular organism communicate with other cells and they act together in coordination. While the long-range interactions between cells are carried through by endocrine or neural signaling, short range interactions are carried primarily through direct cell-cell contact. There are four different types of direct interactions in eukaryotic cells, which are anchoring junctions, tight junctions, desmosomes and gap junctions (Seymour, Tucker, and Leach 2004). They perform various tasks ranging from maintaining attachments between cells to ensuring communication. Among these four junctions, gap junctions are the ones that provide direct link between cytoplasms of adjacent cells. By forming direct linkage between cells, gap junctions allow cells to coordinate their functions (Evans 2002). What differs gap junctions from other three cellular interaction complexes is that gap junctions allow molecules smaller than 1000 Da such as ions, small metabolites and secondary messengers to pass (Loewenstein 1981, Mese, Richard, and White 2007).

The principle component of gap junctions are innexins (Inx) in nonchordate and connexins (Cx) in chordate animals. Although they both form gap junctions, innexins and connexins are not homologous in terms of sequence but their organization and role in the junctions are similar (Mese, Richard, and White 2007, Beyer and Berthoud 2018). There are 21 and 20 Cxs in humans and mouse respectively, 19 of which are orthologs. However, there are connexin genes present only in the mouse (mCx33) or in the human (hCx25 and hCx59) (Laird 2006, Willecke, Eiberger, Degen, Eckardt, Romualdi, Guldenagel, et al. 2002). Since there are multiple isoforms of connexin proteins, two different nomenclature for them have been developed. One of them uses the molecular weight of the connexin proteins, so according to this nomenclature, Cx37 and Cx32 are 37 and 32 kDa, isoforms, respectively. Other nomenclature names connexins according to their amino acid similarities with alpha, beta, gamma, delta and epsilon subgroups.

According to this Cx37 and Cx32 are GJα4 and GJß1, respectively (Willecke, Eiberger, Degen, Eckardt, Romualdi, Güldenagel, et al. 2002). Due to having two nomenclature systems, every connexin has two distinct names (Table 1.1).

Table 1.1. Name of connexins according to both nomenclatures (Kumar and Gilula 1996)

Greek letter nomenclature	Molecular mass nomenclature	Predicted Molecular Mass (kDa)
α1	Cx43	43.0
α2	Cx38	37.8
α3	Cx46	46.0
α4	Cx37	37.6
α5	Cx40	40.4
α6	Cx45	45.7
α7	Cx33	32.9
α8	Cx50	49.6
ß1	Cx32	32
ß2	Cx26	26.5
ß3	Cx31	31
ß4	Cx31.1	31.1
ß5	Cx30.3	30.3

Each of the connexins fit a general structure where connexin polypeptide passes through the lipid bilayer 4 times with both its N and C terminal ends facing cytoplasm and has one cytoplasmic and two extracellular loops (Milks 1988, Yeager 1992). A gap junction is formed when a hemichannel (connexon) of one cell that is made out of 6 connexins, docks end to end with another hemichannel on the plasma membrane of the adjacent cell (Sohl 2004) (Figure 1.1). Subsequent Gap Junction Intracellular Communication (GJIC) is regulated by channel gating via chemicals, pH, and voltage; by modifications such as phosphorylation and ubiquitination of connexins as well as hemichannel insertion, internalization, and degradation (Laird 2006, Leithe 2007, Solan 2009). Hemichannels do not only function within gap junctions; studies revealed that uncoupled hemichannels function through the transfer of molecules between the cytoplasm and the extracellular matrix (Retamal 2007, Laird 2010, Vuyst et al. 2007).

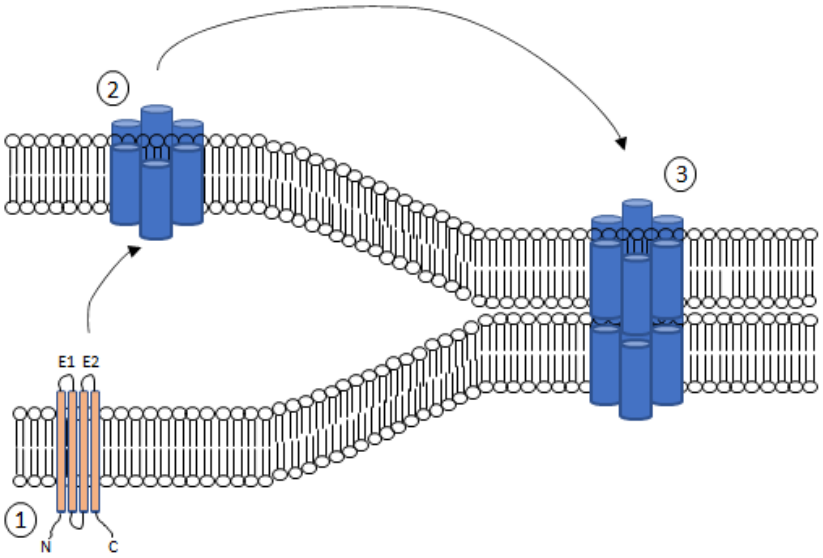

Figure 1.1. Molecular structure of connexins, hemichannels and gap junctions
1-Topology of connexins. Each cylinder represents 1 transmembrane domain. E1 and E2 are extracellular loops. 2- Hemichannel formation. 6 connexins come together to form a ring that is the hemichannel. 3- Gap junctions. Two hemichannels join to form a pore that is the gap junction.

Hemichannels play role in calcium signaling as well as affecting the cells development, proliferation and survival (Schalper, Carvajal-Hausdorf, and Oyarzo 2014). Hemichannels in resting conditions remain closed in the membrane and need to open up to function. This consequently affects the exchange of ions and small molecules that are transferred through the plasma membrane (Aasen et al. 2019).

The types of channels depend on the type of connexins that form the hemichannels. If connexins in different hemichannels that are paired are the same type of connexin, then that the gap junction channel is homotypic. A heterotypic channel is formed when two hemichannels consisting of different types of connexins are paired. Similarly, a heteromeric channel forms by the mixing of multiple connexins within the same hemichannel. If hemichannels in a channel consist of entirely one type of connexins then they are homomeric channels. Since abilities of the gap junctions are defined by connexins, forming different kinds of hemichannels creates versatility to gap junction channels (Figure 1.2).

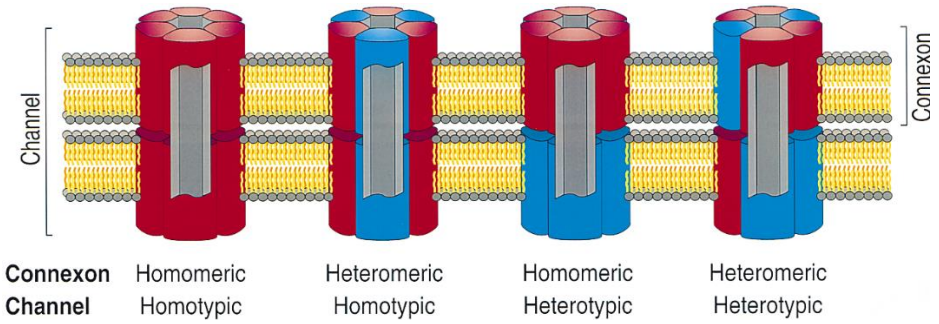

Figure 1.2. Possible arrangements of connexins in hemichannels to form gap junctions. A hemichannel is homomeric if all connexins are the same (all red or all blue), it is heteromeric if it consists both. If the hemichannels are identical, the gap junction channel is homotypic, if they are not then the gap junction channel is heterotypic (Kumar and Gilula 1996).

When their oligomerizations are considered however, it is observed that not all connexins can be in heteromeric hemichannels with other connexins. In fact all heteromeric hemichannels are formed by connexins that are in the same Greek subgroup (Table 1.2). As charges and shapes of connexins affect the rate and type of transfer of molecules, the composition of hemichannels matter a lot (Goldberg, Valiunas, and Brink 2004). Due to different tissues having different requirements, connexin presence in cells differ from tissue to tissue. Eventhough the type of connexin or the tissue is different, what a connexin protein goes through within the cell is similar during the generation of the gap junction channel.

Table 1.2. Connexin combinations that have been reported in literature

Connexin 1	Connexin 2	Reference
Cx26	Cx32	(Stauffer 1995)
Cx26	Cx30	(Ahmad 2003)
Cx37	Cx43	(Brink et al. 1997)
Cx40	Cx43	(He 1999, Valiunas 2000)
Cx43	Cx45	(Martinez et al. 2002)
Cx43	Cx56	(Berthoud et al. 2001)
Cx46	Cx50	(Jiang 1996)

1.2. Life Cycle of Connexins

Connexins function in the cell, similar to every other protein, is directly related to its position in cell (localization) and life expectancy (turnover rate). Biosynthesis, assembly, localization and degradation of gap junctions appear to follow the general secretory pathway similar to almost all membrane proteins. What distinguishes connexins from most of the proteins is their short turnover rate which is less than 5 hours (hrs) (Falk, Kells, and Berthoud 2014).

The connexin gene has a common structure with two exons separated by an intron sandwiched between 5' and 3' untranslated regions (Söhl and Willecke 2004). There are three exceptions to this structure, 5' untranslated region can be spliced and both spliced variants are kept, 5' untranslated region can be spliced and one spliced variant is kept, and an intron interrupts connexin coding region (Salameh 2006) (Figure 1.3). After splicing connexin protein gets synthesized.

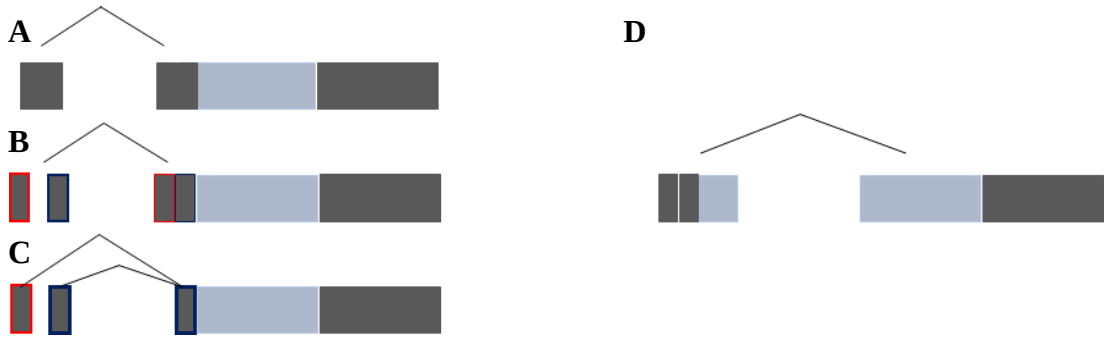

Figure 1.3. Alternate splicing of Connexin genes. A-Both 5'UTRs spliced variants can be kept, B- 5'UTRs spliced consecutive to exon, C- 5'UTRs spliced alternative to exon D- coding region of the connexin can be spliced (Sohl 2004)

Similar to other transmembrane proteins, connexin protein is synthesized at the ribosomes on the endoplasmic reticulum (ER), and integration into rough ER membrane is the first event in the biosynthesis of connexins (Falk 1994, Rahman 1993). Connexin oligomerization occurs sequentially with its insertion into ER and later move to Golgi apparatus where oligomerization gets completed in ER-Golgi network (Falk 1994).

One exception to this is Cx26 which can be inserted to ER membrane both co- and post-translationally, or even directly get inserted to plasma membrane without passing through Golgi apparatus (Ahmad and Evans 2002).

There are two signals that are responsible for connexin recognition and match-up during connexon formation. Predominantly, connexon oligomerization of connexins are determined by the C-terminal peptide sequence that is responsible for connexin subunit recognition. Also there is a selectivity signal located at the amino terminus or at the first transmembrane domain or the first extracellular loop that determines the connexin compatibility for hemichannel formation (Dbouk et al. 2009, Salameh 2006, Willecke, Eiberger, Degen, Eckardt, Romualdi, Guldenagel, et al. 2002).

Following oligomerization, connexons are packaged into vesicles and quickly transported to the cellular membrane with the help of microfilaments and microtubules. (Rook 1990, Laird 1996). Upon insertion to the membrane, connexons might couple with another connexon from a neighboring cell to form gap junctions or remain uncoupled. Due to their short life spans, connexin internalization and degradation happen in quick succession after insertion (Falk, Kells, and Berthoud 2014). Generally, the newly formed connexons are inserted at the periphery of the plaque while the old connexons to be degraded are found in the middle. Internalization occurs using double membrane vesicles that contain either the whole connexon or a single/couple connexins (Thévenin et al. 2013). Following internalization, connexons disassemble into individual connexins, which are further degraded in proteasomes or lysosomes (Jordan 2000) (Figure 1.4). As mentioned before, connexins are dynamic proteins that go through association and disassociation rapidly. Mutations that affect oligomerization or plaque formation would affect this process and therefore might lead to many diseases.

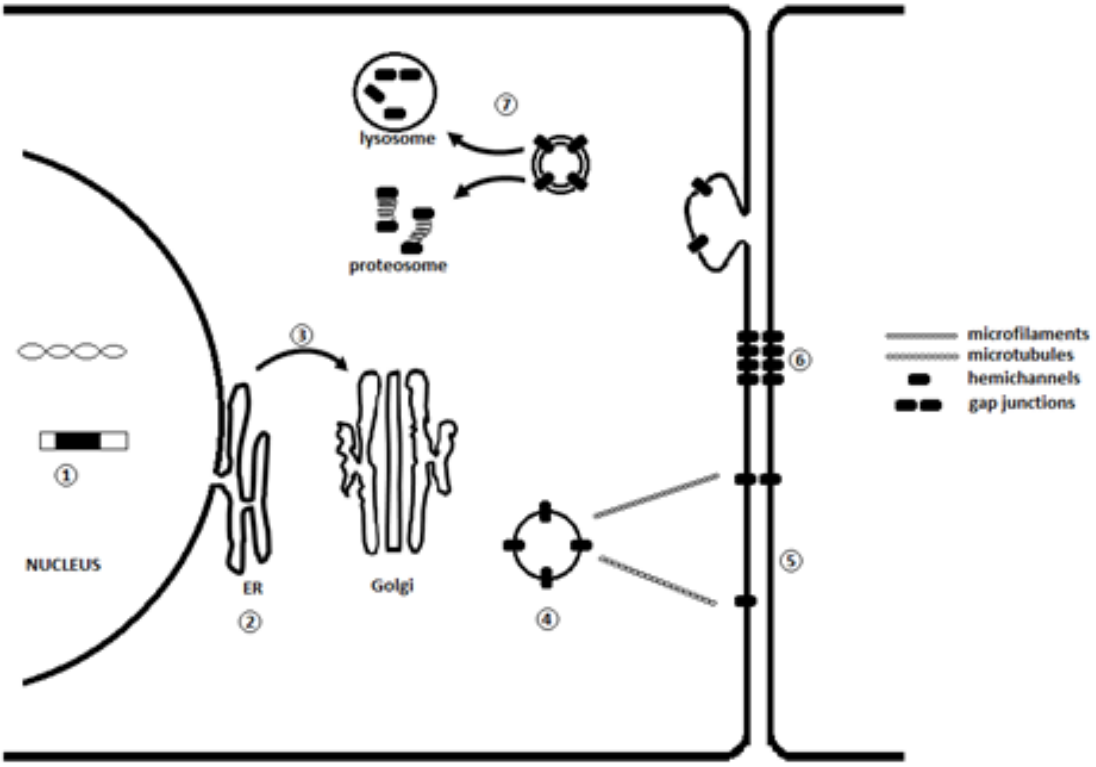

Figure 1.4. Life cycle of gap junctions. 1-splicing of the gene, 2- synthesis of the connexin protein, 3- connexon oligomerization, 4- connexon transport to the membrane, 5- insertion of connexon to the plasma membrane, 6- plaque formation if multiple channels are inserted, 7- degradation of connexins. Modified from Laird, 1996 (Laird 1996)

1.3. Role of Connexins in Human Diseases

Connexins are essential for tissue homeostasis as alterations in connexins due to mutation, aberrant expression or localization can disrupt connexin or GJIC function which can interfere with cellular functions causing diseases (Yamasaki et al. 1999, Cronier et al. 2009, Paul 1995, Sohl 2004). Even though loss of connexins are occasionally compensated by the presence of other connexins, it is not always the case. In those occurrences even a mutation of a single connexin can result in the development of diseases (Srinivas, Verselis, and White 2018, Yamasaki 2018). Disease causing mutations can have different effect on connexin proteins. Mutations might cause proteins to fail quality control and be retained in the Golgi apparatus (Golgi) or go through the endoplasmic reticulum associated degradation (ERAD) (Laird 2006). Proteins might have

a problem with forming functional hemichannels or gap junctions. They might interact with connexins that they normally do not with gain of function mutations (Thévenin et al. 2013). The most commonly seen disease due to connexin mutations is sensorineural hearing loss, which occurs in ~1/2000 births which is due to Cx26 mutations (Chan and Chang 2014). Among other commonly observed diseases, X-linked Charcot–Marie–Tooth disease is X-linked peripheral nervous system disease due to demyelination where Cx32 mutation disrupts continuity in Schwann cells (Bergoffen et al. 1993). Oculodentodigital dysplasia is a complex autosomal dominant disease caused by Cx43 mutation that affects eyes, teeth and fingers, occurs early on in development and spreads to human body later on (Paznekas et al. 2009). Pelizaeus Merzbacher-like disease is an autosomal recessive disease in which mutations on Cx47 gene disrupt heterotypic gap junctions between astrocytes and oligodendrocytes within the central nervous system (Kim, Gloor, and Bai 2013). Mutations on Cx26 cause keratitis-ichthyosis-deafness (KID) where leaky hemichannels dysregulate calcium homeostasis in the epidermis (Sanchez and Verselis 2014). This myriad of disorders shows connexins function in a vast number of tissues and indicates that disruptions in cell function due to mutations in different connexins can lead to different directions (Table 1.3). In addition to these hereditary diseases, dysfunction of connexins play a variety of roles in cancer as well.

1.4. Connexins and Their Function in Cancer

Gap junctions play a key role in tissue development and maintenance in multicellular organisms by connecting adjacent cells. In cancer cells, there is a partial to total loss of coupling capacity among cancer cells and also between cancer cells and normal cells that are surrounding them (Mesnil et al. 2005). Reintroduction of connexins into tumor cells reduces cell proliferation and tumor growth (Eghbali et al. 1991). Also, mice expressing dominant-negative mutations on connexins have increased incidence of tumor onset when exposed to a carcinogen. Despite looking as if connexins have tumor suppressor roles in cancer, they play both tumor suppressive and tumor promoting roles functioning separately or in conjugation with other connexins as part of GJIC or in a GJIC-independent manner.

There are three stages in solid tumor progression; tumor formation in which cells grow uncontrollably, tumor invasion in which cells gain motility and tumor dissemination

in which cells interact with endothelial barriers. Thus, connexins' roles might change in different stages of tumorigenesis and thus they need to be taken into consideration separately (Fig 1.5).

Table 1.3. Connexin expression in disorders (Srinivas, Verselis, and White 2018, Yamasaki 2018).

Gene	Protein	Expressed cell type	Disorder(s)
GJA1	Cx43	Astroglia, endothelium, heart, fibroblast, T cell, B cell, monocyte, macrophage, neutrophil, dendritic cell	Craniometaphyseal dysplasia Erythrokeratodermia variabilis Oculodentodigital dysplasia Palmoplantar keratoderma Syndactyly, type III
GJA3	Cx46	Lung epithelium, eye lens, osteoblast	Cataract
GJA5	Cx40	Endothelium, heart, T cell, B cell, myoblast	Atrial fibrillation
GJA8	Cx50	Eye lens	Cataract
GJB1	Cx32	Microglia, oligodendroglia	Charcot-Marie-Tooth X-linked
GJB2	Cx26	Microglia, hepatocyte, Kupffer cell, stomach, keratinocyte, lung alveolar	Bart-Pumphrey syndrome Deafness Keratitis-ichthyosis-deafness syndrome Vohwinkel syndrome
GJB3	Cx31	Small intestine, colon, skin	Deafness Erythrokeratodermia variabilis
GJB4	Cx30.3	Keratinocyte	Erythrokeratodermia variabilis
GJB6	Cx30	Astrocyte, keratinocyte	Deafness Clouston syndrome
GJC2	Cx47	Oligodendroglia	Leukodystrophy Spastic paraplegia type 44 Lymphedema Pelizaeus Merzbacher-like disease

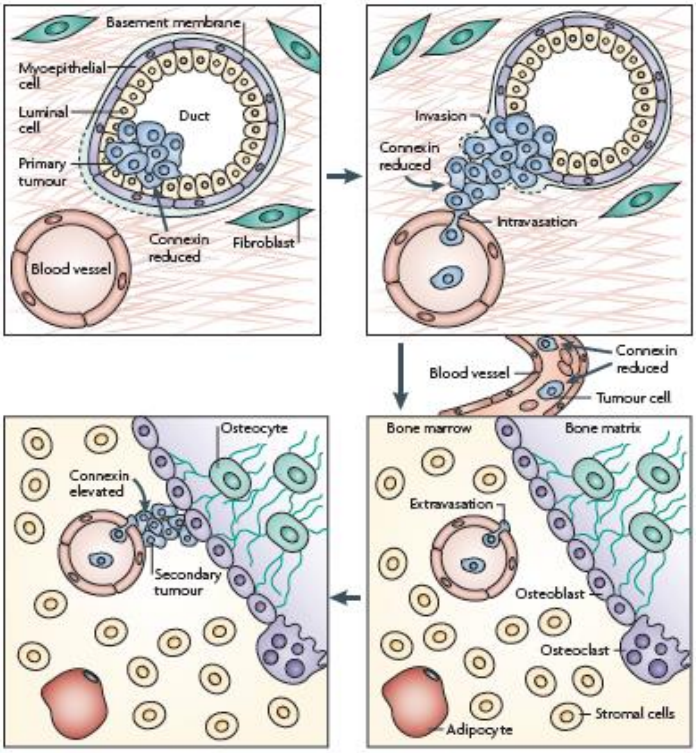

Figure 1.5. Connexin involvement at different stages of breast cancer. At early stages of primary tumors connexins are downregulated and do not form gap junctions. As disease progresses, tumor cells intravasate, disseminate (frequently to the bone) and extravasate to form secondary tumors where connexin expressions were elevated (Naus and Laird 2010).

1.4.1. Function in tumor growth

One of the first changes in tumor formation is an increase in cell proliferation which is closely associated with the cell cycle control. In studies that look at changes in cancer following connexin enhancement, decrease in cell proliferation is one of the frequent observations (Cronier et al. 2009). Connexins influenced the cell cycle, either through reducing the cell cycle progression by changing the length of G_1 and slowing the entry to the S phase in Rin and U2OS cells respectively (Burt et al. 2008, Zhang et al. 2001) or by modulating the expressions of the cell cycle control proteins such as cyclin A, cyclin D1 and cyclin D3, CDK5, CDK6, p21 and p27 in osteosarcoma and retinoblastoma (Zhang et al. 2001, Sánchez-Alvarez et al. 2006). Further, the presence of GJIC is shown to be inversely correlated with cell growth. When GJIC was re-established in tumor tissues, it led to growth inhibition, while blocking GJIC in tumors with GJIC

activity, promoted cell growth (Asadi-Khiavi et al. 2011, Shaw et al. 2007). GJIC is also controlled by molecules functioning in tumor formation and progression. Antitumor agents such as retinoic acid or retinol increased GJIC (Langlois et al. 2008), while in studies performed on rat liver cell line IAR20 and mouse cell line BALBc/3t3 showed that oncogenes such as Ras, v-mos, neu, Src reduced GJIC (Leithe et al. 2009).

The association of gap junctions and tumor growth might not be limited to correlation of tumor growth with loss of gap junctions. Studies in mice showed a correlation with connexin defects with increased susceptibility to certain cancers (Avanzo et al. 2004, Suarez and Ballmer-Hofer 2001). In Fischer 344 rats after chronically treated with 4 carcinogens for 5 weeks, cells showed reduced dye coupling and gap junction numbers with a higher proliferation rate while Cx32 got relocalized to the cytoplasm (Yamasaki and Naus 1996). When mice were treated with urethane 15-17 days after birth, mice with heterogeneously deleted Cx43 developed more and larger nodules compared to wild type mice after 25 weeks (Avanzo et al. 2004). In breast cancer cell lines, blockage of gap junctions inhibit cell growth and apoptosis related genes BCL2 and CYCS (Asadi-Khiavi et al. 2011).

Although connexins play a role in cancer by acting through GJIC, sometimes they acted independently from gap junctions (Duflot-Dancer 1997, Huang et al. 1998, Jiang and Gu 2005). In human glioblastoma cell lines U251 and T9SG, introduction of Cx43 decreased cell proliferation both *in vitro* and *in vivo* while not affecting GJIC at all (Huang et al. 1998). In C6 glioma cells, introduction of Cx32 also reduced tumor growth in mice without affecting GJIC (Bond 1994). In MDA-MB-231 breast cancer cells, neither Cx26 nor Cx43 overexpression creates GJIC, but both connexins represses tumor growth in mice (Qin et al. 2002). In HeLa cells, transfection of mutated Cx26 increased growth while not affecting dye coupling (Duflot-Dancer 1997). On the other hand, transfection of wild type connexin did not induce growth neither *in vivo* nor *in vitro* but improved dye coupling in HeLa cells (Duflot-Dancer 1997). These suggested that connexins role in tumor growth control was independent from their functions in GJIC.

Connexins role in tumor growth goes both ways. When they are functioning in gap junctions, they have growth inhibitory effect while when acting alone, they do seem to be promoting growth (Sinyuk et al. 2018). This might be due to domains of connexins that interact with other connexins or proteins and therefore function in GJIC are different from regions playing a role in tumorigenesis.

1.4.2. Function in motility

Even though primary role of connexins is the exchange of metabolites and signaling molecules between neighboring cells through gap junctions, the non-covalent bond formed between them to form gap junctions also creates adhesive property as an additional effect (Elzarrad et al. 2008, Lin et al. 2002). This also has a role in cancer as adhesive properties of a cell can affect its migration and hence metastasis potentials. Connexins are shown to be involved in adhesion and migration in both normal tissue development and in tumor progression (Kotini and Mayor 2015). Cx32 and Cx43 provides mechanism for adhesion for cell movements during *Xenopus* development (Paul 1995). In C6 malignant glioma cells, Cx43 increases adhesion and migration within GJIC (Lin et al. 2002). In mouse neural cells Cx43 was correlated with migration (Waldo, Lo, and Kirby 1999). Cx26 and Cx43 promotes migration by providing dynamic adhesion between cells for movement in radial glia *in vivo* (Elias, Wang, and Kriegstein 2007).

It is established that upon loss of a connexin, other connexins can compensate the functions of the lost one to a certain extent in mice (White 2003, Bedner, Steinhäuser, and Theis 2012). In mouse retina, Cx36 was found to be functionally replacing Cx45 (Frank et al. 2010). Mice lacking Cx43 dies postnatally due to heart defects but this it can be rescued by Cx32 and Cx40, indicating some functional compensations (Plum et al. 2000). However, loss of Cx43 in neural crest migration cannot be compensated by other connexins, causing heart defects (Lin et al. 2002). Cx43's role in migration might be more important than functioning in neural development since Cx43 has also been identified to have a role in migration in a screen in MCF10A breast epithelial cells, using small interfering RNA (siRNAs) targeting 1081 human genes (Simpson et al. 2008).

Role of connexins in motility is neither always dependent on their functions in GJIC nor they are consistent among all connexins. Transfection with Cx43 but not Cx40 or Cx31 caused HeLa cells to become more invasive than their untransfected counterparts in a non-gap junction dependent manner (Graeber and Hülser 1998). Unlike their role in growth inhibition, gap junctions might have a promoting effect on invasion in non-GJIC way. It was shown that junctionally coupled tumor cells that are able to communicate with the host cells are invasive while, non-coupled tumor cells are non-invasive (Brauner 1990). Also invasion of lung squamous cell carcinoma primary cells is associated with gap junctions and Cx26 expression (Ito et al. 2006). Cx26 expression was low in cells that remain in basal layer but increased in cells that are invading the dermis (Ito et al.

2000). This effect is inhibited and metastatic ability decreased when Cx26 protein was inhibited with oleamide (Ito A 2004). In human liver cancer cell line (HUH7) Cx32 was shown to be associated with invasiveness and motility only when it is accumulated in the cytoplasm and not functioning in GJIC (Li et al. 2007).

Much like for growth control, role of connexins in mobility is complex. Connexins affect motility in some cases together with GJIC but in others independent of GJIC, as well as promoting motility in some cells and inhibiting in others, making the role of GJIC a case specific one.

1.4.3. Function in metastasis

Metastasis is the relocation of a cancer cell from its originating tissue to another tissue using vasculature. There are evidence that connexins may be involved in intravasation and extravasation of cancerous cells as they are required to maintain communication with endothelium (McLachlan, Shao, and Laird 2007). This effect might be due to their role in angiogenesis. In general, presence of connexins have protective effect against angiogenesis since in MDA-MB-231 cells, media conditioned by either Cx26 or Cx43 overexpressing cells upregulated anti-angiogenesis genes MCP-1, CCN1, CCN2 and IL-6 and reduced tubulogenesis and migration in human umbilical vein endothelial cells (HUVEC) cells that grew in it (McLachlan et al. 2006). Just like tumor growth and invasion, connexins' role in metastasis is separate from GJIC (Qin 2003, McLachlan et al. 2006). Independent from GJIC, Cx26 upregulate anti-angiogenesis genes thrombopondin 1 and spock, and downregulate angiogenesis genes CTGF and CYR61 in MDA-MB-453 (Qin 2003).

In HUVEC, overexpression of Cx32 increased tube formation and cell migration while overexpression of Cx37 and Cx43 reduced branching (Okamoto et al. 2014). Cx43 expression in human malignant glioma cells are correlated with increasing tubulogenesis of HUVECs, and VEGF presence in the media of Cx43 expressing cells are found to be higher. In addition, Cx43 found to be functioning in dye transfer, suggesting a GJIC related role of Cx43 in malignant glioma cells (Wei Zhang et al. 2003).

Apart from its effect on angiogenesis, presence of Cx26 is also associated with metastatic capacity (Ito et al. 2006, Ito A 2004). Transfection of Cx26 in non-metastatic melanoma cells transforms them to be metastatic. Also, transfection of dominant-negative

Cx26 mutant to metastatic melanoma cells prevented GJIC and decreased their metastatic capacity (Ito et al. 2000).

Similar to growth and motility, the same connexin might have different effects on various types of cell or different connexins might have different effects on the same cell when it comes to intravasation and extravasation of cancerous cells.

1.5. Cx32 and Cancer

Role of Cx32 in cancer cells differ in various cancers. In a hybrid of A549 lung cancer cell line and HUVEC (EA.hy926), Cx32 promotes tube formation, wound healing, vascular sprouting, and cell migration, which can be concluded as promoting angiogenesis (Okamoto et al. 2014). In hepatocellular carcinoma, Cx32's effect is location dependent as Cx32 mediated GJIC downregulated cell motility whereas cytoplasmic Cx32 increases invasion and metastasis in mice (Li et al. 2007) . This location dependent effect has also been observed in prostate, gastric and colon cancers (Jee et al. 2011, Kanczuga-Koda et al. 2010, Mehta 1999). In gastric carcinoma, Cx32 arrested cell cycle at G_1 stage by increasing expression of $p21^{Cip1}$ and $p27^{Kip1}$ (Jee et al. 2013). Also in murine gastric carcinoma, Cx32 expression and proliferation is inversely related (Jee et al. 2011). When prostate cancers at different grades are examined, it was observed that Cx32 expression decreases as tumor grade advances (Mehta 1999). When transfected to lung carcinoma cells, Cx32 reduced anchorage independent growth, invasiveness and development of tumors in a xenograft model while inducing contact inhibition (Hada 2006).

1.6. Breast Cancer

Breast cancer is one of the most commonly observed cancer types and the second leading cause of cancer-related death in women, affecting one in every eight of them during their lifetimes in developed and developing countries (Ferlay, Parkin, and Steliarova-Foucher 2010, Siegel, Miller, and Jemal 2020). Though primary tumor of the breast cancer is relatively benign, the leading cause of death due to breast cancer is the metastasis to different tissues, primarily the lungs, the bone, the brain and the liver (Weigelt, Peterse, and van't Veer 2005). Metastasis begins with tumor cells separating from the primary

tumor body, crossing the basal membrane into the blood stream (intravasation), circulate in the blood stream, leave the blood stream (extravasation), settle in another tissue and proliferate (Yang et al. 2011) (Fig 1.6).

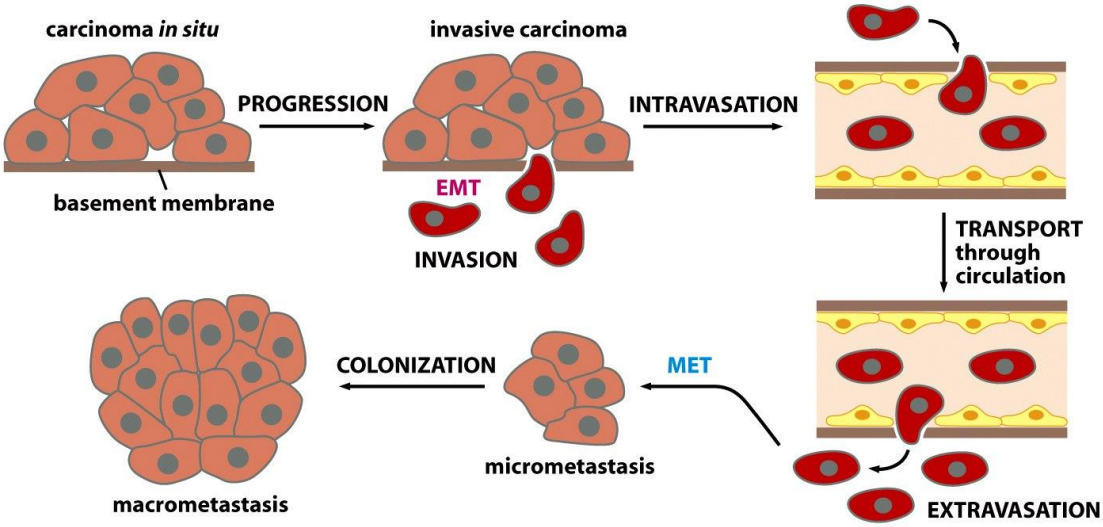

Figure 1.6. The process of metastasis. Cells go through EMT, intravasation, extravasation and MET in order to metastasize to a tissue. Cells first secrete enzymes to degrade basement membrane. Escaping cells go through EMT which enables cells to intravasate into blood vessels, circulate and extravasate. Afterwards cells go through MET to colonize to their new location (Weinberg).

In order for cells to transfer between tissue and blood they need to transform. The transformation process that they go through before entering blood stream is known as epithelial to mesenchymal transition (EMT) and the process they go through after they leave the blood stream is called mesenchymal to epithelial transition (MET) at the metastasis site (Tsai and Yang 2013). For EMT, cell goes through certain changes, downregulating some attachment related proteins, expressing certain transcription factors and finally upregulating structural proteins. Among these molecules, there are cell surface molecules such as mesenchymal marker N-cadherin and epithelial markers E-cadherin and Zona Occludens 1 (ZO-1). Although both N-cadherin and E-cadherin share similar structures and have similar roles in cell-cell interactions, they are active in different types of cells. Mesenchymal marker Vimentin is a cytoskeletal molecule. Slug, Snail and Zeb2 are transcription factors playing role in EMT. Zeb2 is a E-cadherin repressor. Snail and Slug are part of Snai family that along with other functions downregulate E-cadherin,

decrease the expression of occludins, increase the expression of mesenchymal marker fibronectin and inhibit proliferation (Zeisberg and Neilson 2009) (Fig 1.7).

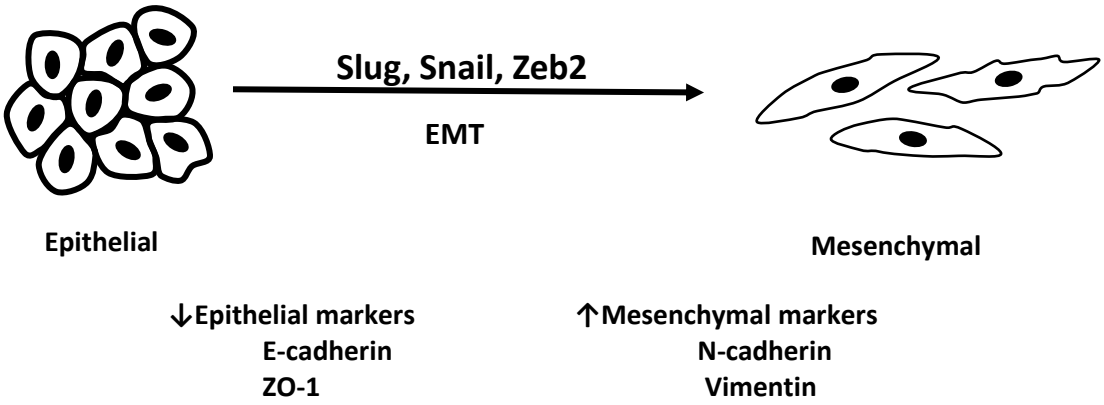

Figure 1.7. EMT markers used in this study. E-cadherin is an epithelial cell adhesion molecule, ZO-1 is a tight junction molecule. Both of these are epithelial markers. N-cadherin is mesenchymal cell adhesion molecule, Vimentin is an intermediate filament. Both of these are mesenchymal markers. Slug, Snail and Zeb2 are transcription factor. All three of them are mesenchymal markers.

1.7. Connexins and Breast Cancer

Among many molecules, connexins and gap junctional communication were shown to have role in breast cancer. There are three connexin isoforms detected in the mouse breast; Cx26, Cx32 and Cx43 (McLachlan, Shao, and Laird 2007), while normal human breast tissue expresses Cx26 and Cx43, Cx32 is not observed (Monaghan et al. 1996). In breast cancers it was found that both Cx26 and Cx43 are transcriptionally downregulated and gap junctions are absent. Upon individual transfections to MDA-MB-435 breast cancer cells, each of them act as tumor suppressors and restore differentiation potential (Hirschi et al. 1996). It was also shown that disruptions in GJIC causes breast cancer cells MDA-MB-361 to metastasize (Navolotski et al. 1997). In addition to that, Cx26 and Cx43 regulate molecular pathways linked to EMT and angiogenesis (McLachlan et al. 2006).

Despite not present in normal human breast tissue, cytoplasmic Cx32 was expressed nearly 40% of breast cancers while their non-tumorigenic neighbors do not

express Cx32 (Kanczuga-Koda et al. 2007). Cx32 expression increased for lymph node metastases compared to primary tumors and Cx32-negative primary tumors developed Cx32-positive metastases, suggesting a role for Cx32 in breast cancer progression (Kanczuga-Koda et al. 2007).

1.8. Breast Cancer Types

Breast cancer is a heterogeneous and complex disease where there are pathological, clinical and molecular differences between different breast cancers. Breast cancers are divided into five subtypes according to their molecular markers and the presence of receptors such as estrogen (ER), progesterone (PR) and HER2. Those five subtypes are as follows; Her2-enriched, luminal A, luminal B, basal-like , and unclassified (normal breast-like) subtypes (Banerji et al. 2012). Her2-enriched tumors are Her2 receptor positive, independent from their ER/PR status. They account for 12-20% of invasive tumors. Luminal A tumors constitute 30-40% of invasive tumors. They are generally ER/PR positive while Her2 negative. Luminal B tumors constitute 20-30% of invasive tumors. They are generally ER positive while PR/Her2 negative. Basal-like tumors are triple negative tumors that express none of the receptors. They account for 15-20% of all invasive tumors. Unclassified tumors are ER and/or PR positive, Her2 negative. They express low amounts of Ki-67 protein, which is an indicator for how fast tumor cells grow (Dai et al. 2016, Fragomeni, Sciallis, and Jeruss 2018).

1.9. Aim of the Project

Role of Cx32 in breast cancer is not known. Especially considering that Cx32 expression in breast cancer coincides with tumor formation and metastasis (Kanczuga-Koda et al. 2007), its role should be explored. The aim of this study is to provide information about the role of Cx32 in breast cancers with varying levels of aggressiveness and correlate this role to the functionality of gap junctions and localization of Cx32.

Breast cancer cell lines used in this study are Hs578T and MCF7 cell lines. Hs578T breast cancer cell line is a basal triple negative aggressive cell line. The other cell line is MCF7 cell line which is a luminal, ER(+) cell line that is mildly aggressive.

CHAPTER 2

MATERIALS and METHODS

2.1. Maintenance and Selection of Cell Lines

In this section methods that are used in relation to cell lines are described. The conditions that the cells were grown, and their maintenance techniques are explained. Also, generation of the transfected and infected cell lines were explained.

2.1.1. Maintenance of Cell Lines

Both MCF7 and Hs578T cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Cat# 41966-029) supplemented with 10% Fetal Bovine Serum (FBS) (BI, Cat# 04-127-1A) and 1% penicillin/streptomycin (Gibco, Cat# 15140-122), with 10μg/ml insulin (Sigma, Cat# I1882) only for Hs578T at 5% CO2 and 37°C.

293T and NIH3T3 cells were briefly cultured for virus production. 293T cells were grown in high glucose DMEM with 10% FBS and 1% penicillin/streptomycin. NIH3T3 cells were grown in high glucose DMEM with 10% Newborn Calf Serum (NBCS) (Life Technologies, Cat# 16010159) and 1% penicillin streptomycin.

All cells were cultured in appropriate tissue culture plates and for passaging, they were trypsinized with 0.05% Trypsin/EDTA (BI, Cat# 03-052-1B) solution for 4 minutes following with 3 minutes of centrifugation at 1200 rpm at room temperature.

2.1.2. DNA Constructs and Their Introduction into Cells

Cx32 expressing cell lines were achieved through both by transient transfection and by stable infection. While transiently transfected cell lines were used right away, stable cell lines went through viral infection, following by antibiotic selection.

For transient transfection, Cx32 gene was cloned into pCS2+ mammalian expression vector. Cells (300000 cell/well in 6-well plate for MCF7 cells and 200000 cells/well for Hs578T cells) were transfected with FuGENE® (Promega, Cat# E2311)

transfection reagent with 1μg DNA: 6μl transfection reagent ratio. After transfection, cells were incubated for 48 hrs until experimentation.

For infection, Cx32 containing pLenti-GIII-CMV-GFP-2A-Puro lentiviral vectors (Abm, Cat# LV169789) and its empty vectors were used. Virus was produced in 293T cells using pMD2.VSVG for envelope and pCMVdR8.74 for packaging vectors and medium containing viral particles were collected at 48 and 72 hrs after transfection. Their titration for optimizing the virus concentration was performed using NIH3T3 cells. Afterwards, MCF7 and Hs587T cells were infected and 72 hrs post infection, selection with 2μg/ml puromycin was performed until the cells in mock has completely killed.

2.2. Proliferation Related Methods

In the following section, methods that are related with cellular proliferation are explained. Methods both directly determine cellular proliferation (BrdU, Colony Formation Assay), and that can be inferred for proliferation (Tryphan Blue Assay, MTT, Cell Cycle Analysis) are described.

2.2.1. Tryphan Blue Assay

Cells were seeded in the plates at 50000 cells/well for Hs578T cells and 100000 cells/well for MCF7 cells. At days 1, 3 and 7, cells were trypsinized, resuspended in 1 ml medium making the final volume of liquid 1.5 ml. Cells were counted using a hemocytometer and tryphan blue (Gibco, Cat# 15250). Numbers were later normalized to initial seed number and resuspension volume.

2.2.2. MTT

After seeding 2500 cells/well for Hs578T and 5000 cells/well for MCF7 to 12 well plates, cells were incubated for 10 days. On days 1, 3, 5, and 7; cells medium was replaced with 500μl fresh medium and 50μl MTT (Amresco, Cat# 0793) were added to all wells and cells were left to incubate for 4 hours. After 4 hours, medium was removed and 300μl of DMSO was added to each well, left to shaking for 30 min in dark. After

shaking, all wells were divided into three for technical replication and their light absorbance at 550nm was measured.

2.2.3. BrdU

Cells were seeded 100000 at 6 well plates on cover glasses. After 24 hrs, BrdU (APC, Cat# 519000019BK) was added at 20μM concentration and cells were incubated for 2 hrs. Afterwards, cells were washed with 1X PBS for 3 minutes twice. Then, cells were fixed with 4% PFA for 20 min at room temperature, washed with 1X PBS three times for 5 minutes. Cells were incubated with 1.5M HCl for 30 min and washed again three times for 5 min. Cells, later were blocked in blocking solution (5%NHS, 0.2% Triton X-100 in 1X PBS) for 1 hr and incubated with α-BrdU (Abcam, Cat# ab221240) in antibody solution (2%NHS, 1% Triton X-100 in 1X PBS) at 1:100 dilution overnight at room temperature. Cells were washed with 1X PBS three times for 5 minutes and incubated 1 hr with DAPI at 1:1000 dilution in antibody solution. After a final wash of 1X PBS three times for 5 minutes, coverslips were mounted on the slides. Images were taken with florescent microscopy and analyzed using ImageJ software.

2.2.4. Colony Formation Assay

Cells were seeded 500 cells/well to 6 well plates and incubated for a week. Afterwards formed colonies were fixed with 100% methanol for 20 minutes and washed with ddH_2O. Fixed cells were stained with 5% crystal violet (Amresco, Cat# 0528) for 10 minutes and washed three times with ddH_2O. Cells were left to air dry overnight. Images of the colonies in 78.5 mm^2 area were taken with brightfield microscopy and colonies were counted.

2.2.5. Cell Cycle Analysis

In order to determine cell cycle phases, cells were stained with propidium iodide and analyzed through flow cytometry. After cells were trypsinized and centrifuged for 10 minutes at 1200 rpm at room temperature. Pellets were then resuspended with 1ml cold PBS and 4 ml 100% ice cold ethanol. Cells were then stored at -20°C up to a month. On

the day of the analysis, cells were centrifuged 10 minutes at 1500 rpm following by 1 min at 2000 rpm at 4°C. Supernatants were aspirated and pellets were resuspended with 1 ml PBS and suspension was transferred to Eppendorf tubes. Cells were then centrifuged for 10 minutes at 1500 rpm at 4°C. Pellets were resuspended with 200µg/ml RNase A (Sigma, Cat# R6148) in 200µl 0.1% Triton X-100 PBS. Cells were later incubated at 37◦C for 30 minutes. Finally, 1mg/ml Propidium iodine (PI) (Life Technologies, Cat# T3605) was added on cells and incubated for 15 minutes at dark. Cells were analyzed by BD FACS Canto flow cytometry.

2.3. Channel Function Related Methods

In the following section, methods that are related with channel functions are described. Both hemichannel activity and gap junctional communication determination is explained.

2.3.1. Dye Uptake

For dye uptake assay, cells were incubated at 37°C in 1 ml DMEM for 20 minutes. After incubation, medium was aspirated, and cells were washed with 1X PBS. 0.5mg/ml neurobiotin in 1X PBS (Vector, Cat# 1120) was added on the cells and incubated for 10 minutes. The cells were washed with medium three times for 10 minutes. Afterwards cells were fixed with 4% PFA for 15 min at room temperature and permeabilized with 0.1% Triton X-100/ 1X PBS for 15 min at room temperature. After permeabilization, they were blocked with 3% BSA in 0.1% Triton X-100/ 1X PBS overnight at 4°C. Next day cells were incubated with rhodamine- conjugated streptavidin (LSG, Cat #21724, and DAPI at 1:500 dilutions for 1 hour at room temperature. Cells were again washed with 1X PBS three times and stored in 4°C at dark in PBS. Blockage of hemichannels were achieved through using 100µM Carbenoxolene (CBX) for every step until fixation.

Cells were observed using Olympus® IX83 florescent microscope using FV1200 Quickstart florescent unit and images were obtained using cellSens program (Olympus) and analyzed in ImageJ (NIH) software.

2.3.2. Scrape Loading

For scrape loading, once cells had achieved desired confluency, they were washed with 1X PBS. 500µl of neurobiotin (0.5mg/ml final concentration diluted in 1X PBS) was given to the cells and 3 cuts/well was performed on the cells with a blade. After 10 minutes of incubation at 37°C, medium was added and incubated again for 20 minutes. Cells were washed with 1X PBS for 10 minutes three times. Afterwards cells were fixed with 4% PFA for 15 min at room temperature and permeabilized with 0.1% Triton X-100/ 1X PBS for 15 min at room temperature. After permeabilization, cells were blocked with 3% BSA in 0.1% Triton X-100/ 1X PBS overnight at 4°C. Next day cells were incubated with rhodamine- conjugated streptavidin at 1:500 dilution, and DAPI at 1:500 dilution for 1 hour at room temperature. Cells were again washed with 1X PBS three times of 10 minutes each and stored in 4°C at dark in PBS. Blockage of gap junctions were achieved by using 100µM CBX for every step until fixation.

Cells were observed using Olympus IX83 microscope using FV1200 Quickstart florescent unit and images were obtained using cellSens program and analyzed using ImageJ and GraphPad® Prism softwares.

2.4. Visualization and Quantification Related Methods

In this part, methods that showed the presence of a molecule (RNA/protein) within a cell are explained.

2.4.1. Immunostaining and Florescent Imaging

For immunostaining experiments, Hs578T and MCF7 cells were seeded on glass coverslips and transfected as described in part 3.3. For immunostaining, cells were washed with 1X PBS, and fixed with 4% Paraformaldehyde (PFA) for 20 min at room temperature. Cells were then permeabilized with 0.1% Triton X-100/ 1X PBS for 15 min at room temperature and blocked with 5% bovine serum albumin (BSA) in 0.1% Triton X-100/ 1X PBS for 1h at room temperature. Cells were incubated with rabbit anti-Cx32 antibody (Invitrogen, Cat# 345700) at 1:200 dilution for 1h at room temperature. After three washes of 10 minutes with 1X PBS, cells were incubated with florescent dye-

conjugated anti-rabbit antibody (Invitrogen, Cat# A21428) at 1:200 dilution, and DAPI (Sigma, Cat# D95242-10MG) at 1:1000 dilution for 45 minutes at room temperature. Cells were again washed with 1X PBS three times for 10 minutes and mounted on a slide using mounting medium and fixed using nail polish.

For colocalization experiments, cells were fixed, permeabilized and blocked. Afterwards, cells incubated with mouse anti-Golgin97 antibody, a Golgi apparatus marker (Invitrogen, Cat# A21270) as well as rabbit anti-Cx32 antibody and washed with 1X PBS. As secondary antibody for Golgi florescent dye-conjugated anti-mouse antibody (Invitrogen, Cat# A11017) at 1:200 dilution was used. For membrane staining, cells were incubated with Rhodamine conjugated-Wheat germ agglutinin (WGA) (Vector, Cat# RL-1022) in 1X PBS in 1:500 dilution for 30 minutes in 4°C and fixed with 4% PFA. Remainder of the immunostaining protocol was followed afterwards.

Cells were observed using Olympus IX83 microscope using FV1200 Quickstart florescent unit and images were obtained using cellSens program.

2.4.2. Semi quantitative PCR (RT-qPCR)

To determine mRNA expression levels of genes of interest, semi-quantitative real time RT-qPCR was performed. In order to isolate RNA, cells were flash-frozen in liquid nitrogen. Total RNA was isolated from frozen cells using Pure-link RNA Mini Kit (Invitrogen, Cat#12183018A) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1µg isolated total RNA by using Fermentas First Strand cDNA Synthesis Kit (Thermo Scientific, Ca#K1622). SYBR Green based RT-qPCR was performed in 96 well plates with 0.75µl cDNA, 1µl forward and reverse primers (100µM) (Table 2.1), 2.25µl dH_2O and 5µl SYBR Green (Thermo Scientific, Cat# K0252). Reaction was done in Roche LightCycler® 96 (Table 2.2)

Table 2.1. Forward and reverse primer sequences used in RT-qPCR

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Connexin 26	ctgcagctgatcttcgtgtc	aagcagtccacagtgttg
Connexin 32	ggcacaaggtccacatct	gcatagccagggtagagc
Connexin 43	gtgcctgaacttgccttttc	ccctcccagcagttgagtagg
GAPDH	gaaggtgaaggtcggagtca	aatgaaggggtcattgatgg

Table 2.2. Program used for qPCR

Name	Time	Temperature	Cycles
Preincubation	10 min	95°C	1
Amplification	30 sec	95°C	45
	30 sec	60°C	
	30 sec	72°C	
Melting	10 sec	95°C	1
	60 sec	65°C	1
	continuous	97°C	continuous

2.4.3. Protein Isolation and Western Blotting

Protein was isolated in two ways. Proteins were isolated from either flash frozen cells or from fresh cells. For flash freezing; cells were washed with 1X PBS twice and frozen in liquid nitrogen and stored in -80°C. Afterwards they were scraped from the plate using a scraper and 300µl lysis solution (10mM Tris-HCl, 1 mM EDTA, 0.1% Triton X-100, 1% protease inhibitor and 0.1% DTT). For fresh cell isolation cells were trypsinized and centrifuged. After centrifugation, cells were suspended into 1ml PBS and transferred into an Eppendorf tube. Afterwards, cells were centrifuged at 1200 rpm for 5 min and re-suspended with 300µl lysis solution. Lysate were then passed through insulin syringe 10 times and incubated for 20 min on ice for both protocols. The samples were centrifuged at 13000 rpm for 20 min at 4°C and supernatant was transferred into a fresh tube which was stored at -80°C.

For Western blotting, 15 % resolving gel for connexins and 10% resolving gel for EMT markers and 5 % stacking gel for all were used. Equal amounts of protein were incubated at 95°C for 5 min with 5 µl loading dye (250 mM Tris-HCI, 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, 0.02% Bromophenol Blue). Afterwards, the samples were run at 20 mA for one and half hours. The gel was transferred to a nitrocellulose membrane at 250 mA for 120 min. The membrane was blocked for 2 hours with 5 % milk in 1X Tris-Buffered Saline and Tween 20 (TBS-T) solution. Membrane was incubated with proper primary antibody at 1:1000 ratio within 5 % milk in TBS-T solution overnight at 4°C. Next day membrane was washed three times with TBS-T for 10 mins each. Afterwards proper secondary antibody at 1:2500 dilution was incubated at room

temperature for 2 hours (Table 2.3). After incubation, membrane was washed three times with TBS-T for 10 mins each. The image was taken with SuperSignal® West Pico Rabbit IgG Detection Kit (Thermo, Cat#34077). For loading control, mouse anti-γ-tubulin primary (Sigma, Cat# T6557) antibody at 1:1000 dilution and HRP-conjugated anti-mouse secondary antibody (Dako, Cat# 00071312) at 1:2500 dilution was used. Protein levels were normalized to γ-tubulin using ImageJ software.

Table 2.3. Antibodies used in Western blotting

Antibody	Type	Cat#
Cx32 primary	Rabbit	Invitrogen, Cat# 70-0600
Cx26 primary	Goat	Abcam, Cat# ab59020
Cx43 primary	Rabbit	Invitrogen, Cat# 710700
γ-tubulin primary	Mouse	Sigma, Cat# T6557
Vimentin	Rabbit	Cell signaling, Cat# 5741P
Slug	Rabbit	Cell signaling, Cat# 9585P
E-Cadherin	Rabbit	Cell signaling, Cat# 3195P
N-Cadherin	Rabbit	Cell signaling, Cat# 13116P
ZO-1	Rabbit	Cell signaling, Cat# 8193P
Zeb-2	Rabbit	Merck, Cat# ABT332
Secondary	anti-mouse	Dako, Cat# 00071312
Secondary	anti-rabbit	Thermo, Cat# 31460
Secondary	anti-goat	Pierce, Cat# 31460

2.5. Aggressiveness Determining Methods

In this section methods that are related to determination of tumor cell aggressiveness are described. Methods that measures anchorage independent growth, cellular motility or invasiveness are explained.

2.5.1. Soft Agar Assay

1.5 ml 0.5% agar in medium (Difco, Cat# 214220) set at the bottom of 6 well plates. Once it polymerized, 1.5 ml of 0.35% agar with 30000 cells were placed on top of the 0.5% agar. 1.5 ml medium was placed as the topmost layer. The medium was changed once a week until colonies of proper sizes were developed. To prepare for imaging, colonies were stained with 0.05% crystal violet and 5 stacks of 25 frames placed in a 5x5 grid was taken. The images were analyzed using imageJ software.

2.5.2. Wound Healing Assay

In order to determine migration potential of cells, wound healing assay was performed. Cells were seeded in 12 well plates at 900000 cells/ well for MCF7 cells and 450000 cells/well for Hs578T cells. After 48 hrs incubation, 2 mg/ml mitomycin was added in serum-free medium and waited for 2 hrs. After 2 hours, a wound was created with a 10μl pipette tip. Afterwards, cells were washed with 1xPBS and incubated in 1% serum containing starvation medium for 48hrs. During incubation pictures were taken at every hour. Size of the open area was determined by using ImageJ software.

2.5.3. Invasion Assay

In order to determine invasion abilities of cells, invasion assay using Boyden chambers was performed. Chambers were inserted in 24 well plates and were coated with 40μl 1:6diluted Matrigel in serum-free medium. Cells were seeded on the Matrigel at 50000 cells/ well for MCF7 cells and 75000 cells/well for Hs578T cells in serum free medium. Chambers were then inserted into 700μl medium with serum for cells to invade through. After 24 hrs, Matrigel was cleaned and invaded cells were stained with DAPI. Well pictures were taken by Olympus IX83 microscope using FV1200 Quickstart florescent unit and images merged together in cellSens program.

2.6. Statistical Analysis

Two-tailed unpaired t-test was applied to determine statistical significance between GFP control and Cx32 experimental groups. P-values less than 0.05 was accepted as significance threshold. All results were expressed as ± standard deviation (S.D). Statistical significance was shown as $p<0.05$ (*), $p<0.01$(**), $p<0.005$ (***) and $p<0.001$(****) levels.

CHAPTER 3

RESULTS and DISCUSSIONS

3.1. Presence and Overexpression of Cx32 in Cells

For successfully overexpressing the Cx32 gene, baseline levels of endogenous Cx32 in both Hs578T and MCF7 cells were needed to be determined. For this, expression of Cx32 on mRNA and protein levels were verified using RT-qPCR and Western blotting, respectively. Both RT-qPCR (Fig 3.1A) and Western blot (Fig 3.1B &C) showed that Cx32 is expressed in MCF7 cells while Hs578T did not show significant Cx32 expression.

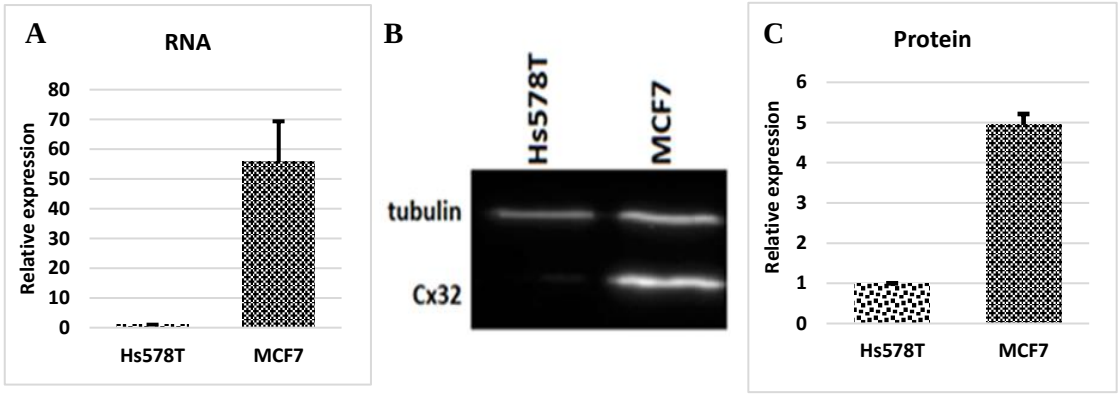

Figure 3.1. Relative Cx32 expression of cells. (A) mRNA levels of cells (n=3) (B) Representative image of protein levels of cells, (C) quantification of protein levels in cells (n=3).

In order to confirm the RT-qPCR and Western blot results in cells, both cells were immunostained against Cx32. Hs578T cells showed slight amount of Cx32 protein, which confirmed mRNA and protein results. MCF7 cells, on the other hand, showed endogenous Cx32 expression which also did confirm previous results, but no Cx32 gap junctional plaques were observed between adjacent MCF7 cells (Fig.3.2).

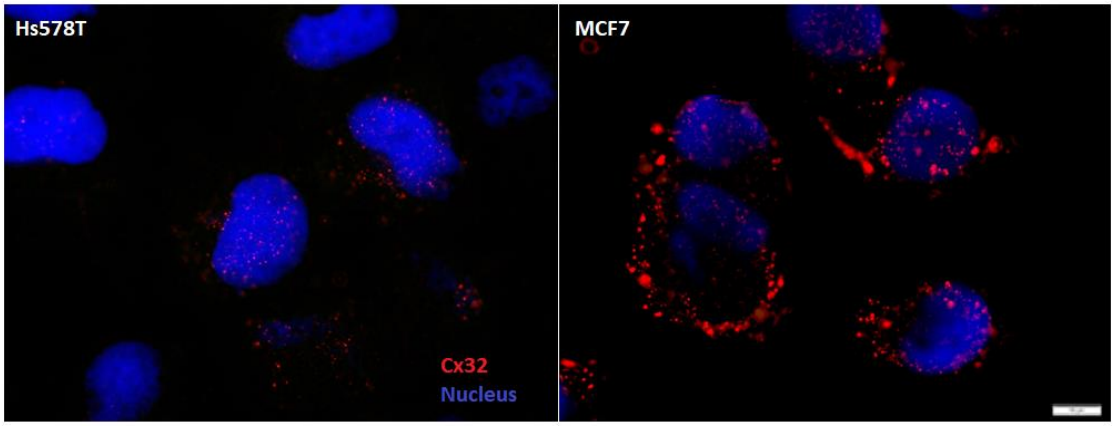

Figure 3.2. Immunostaining of cells. Images taken with 100x magnification (scalebar 10μm.) Red is Cx32 and blue is nucleus.

In order to prepare cells for long term experimentation, stable cell lines were established through viral infections. Both MCF7 and Hs578T cells were infected with Cx32 and GFP containing empty vector (control) as explained in section 2.1.2. Protein and mRNA levels of Cx32 in the infected cells were verified by performing RT-qPCR and Western blot analysis (Figure 3.3). mRNA results showed no significant difference in expression of Cx32 between uninfected and empty-vector infected cells for both cell types (data not shown). There was 274-fold and 180-fold increase in Cx32 expression in Cx32 infected Hs578T and MCF7 cells, respectively. Furthermore, protein analysis indicated that Cx32 levels in Hs578T cells significantly increased while they remained the same for MCF7 cells. The fact that the increase of Cx32 mRNA in MCF7 does not reflect in protein levels might suggest a compensation mechanism at protein level for Cx32 in these cells. Since most of the studies about endogenously and exogenously expressed connexins are done to see heterotypic vs homotypic communication (Mesnil et al. 1995, Eghbali, Kessler, and Spray 1990), there is not much information about dosage compensation for endogenous connexins. It is found that hemichannels made by exogenous Cx43s has higher channel activity than endogenous Cx43 (Barrio et al. 1991) and it is generally believed that endogenous connexins prevent exogenous connexins to be expressed, thus to observe exogenous connexin expression endogenous connexins need to be suppressed (2001). Knowing that there are compensation mechanisms that cover the absence or mutation of a connexin by another connexin (Bedner, Steinhäuser,

and Theis 2012), it is possible for there to be a compensation mechanism for exogenous connexins that are already endogenously expressed in cells.

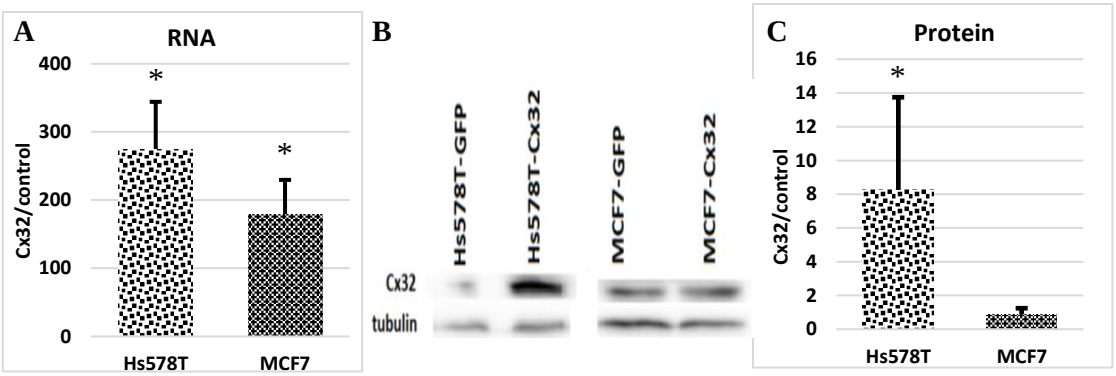

Figure 3.3. Protein and mRNA levels of Cx32 in infected cells. Cx32 expression was obtained by normalizing Cx32 infected cells to control cells (A) mRNA levels in infected cells. (n=3, * $p<0.05$) (B) Representative image of protein levels of Cx32 in infected cells, (C) quantitative comparison of protein levels in infected cells. Error bars represent S.D (n=3; * $p<0.05$).

3.2. Effect of Cx32 on Proliferation of Cells

Change in proliferation rate is one of the most common effects of connexin introduction or exclusion, thus with Cx32 overexpression, changes of proliferation in both cells needed to be addressed. In order to evaluate the effect of Cx32 infection on the viability of the cells, MTT assay was conducted on both control and Cx32 infected cells. It seems that both Cx32 infected cells have slightly higher cell viability than control ones but while in Hs578T cells, the difference was statistically significant starting from day 3, the difference in MCF7 cells was only statistically significant on day 7 (Figure 3.4).

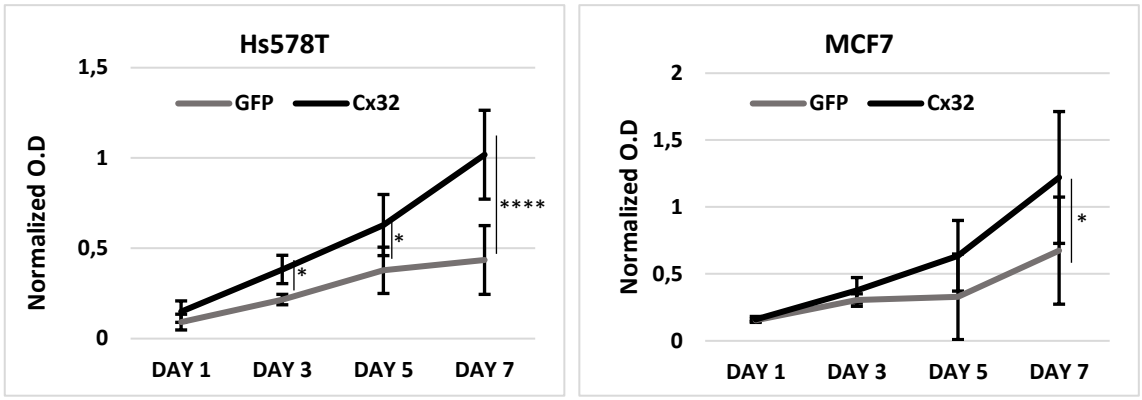

Figure 3.4. MTT results for infections for Hs578T and MCF7 cells. Graph obtained by normalizing O.D of Cx32 infected cells to control cells. Error bars represent S.D. (n=9; * $p<0.05$, **** $p<0.001$)

MTT is an assay for cytotoxicity, and the inference about cellular proliferation arises from the lack of cytotoxic agents. Even though MTT gives an idea about proliferation, it is not a preferred method to assess proliferation as it does not directly measure it. In order to confirm MTT results, cells were directly counted at regular intervals to determine the changes in their proliferative rates (Figure 3.5). Similar to MTT results, Hs578T cells showed increased proliferation with Cx32 infection, but results were only significant at day 7. On the other hand, consistent with the MTT results difference between control and Cx32 cells for MCF7 cells were less significant than Hs578T cells and the increased proliferation was not statistically significant.

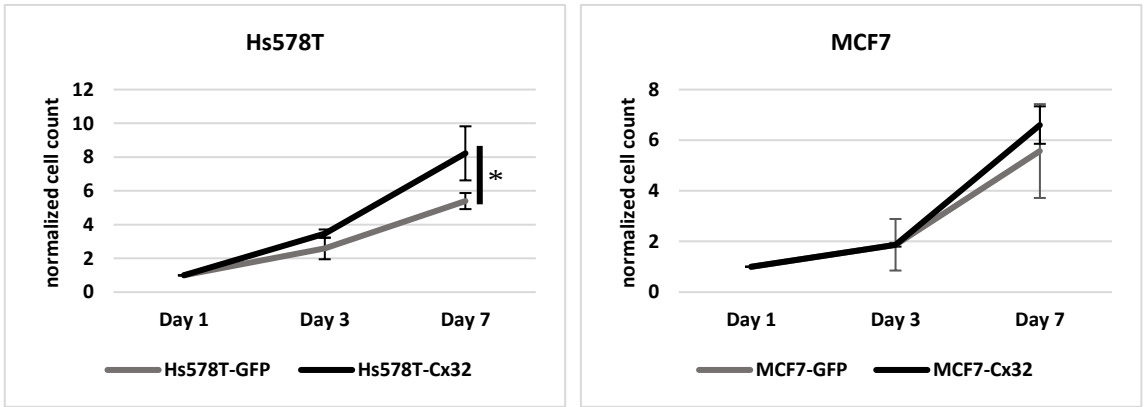

Figure 3.5. Cell counts in 1 ml volume normalized to day 1. Error bars represent S.D (n=3; * $p<0.05$)

Even though tryphan blue assay show proliferation, it does not show dividing cells directly. Therefore, we still infer proliferation from the change in total cell numbers. It is more direct than MTT, but still shows proliferation indirectly. In order to show proliferating cells directly BrdU incorporation assay was utilized. In the assay, proliferating cells during two-hour period were treated with BrdU, and the ratio of dividing cells to total cells were taken. The results showed increased proliferation in both Hs578T and MCF7 cells. Hs578T cells proliferation significantly increased from 1.62% to 4.60% while MCF7 proliferation increased from 2.97% to 3.80% but this increase was not significant (Figure 3.6).

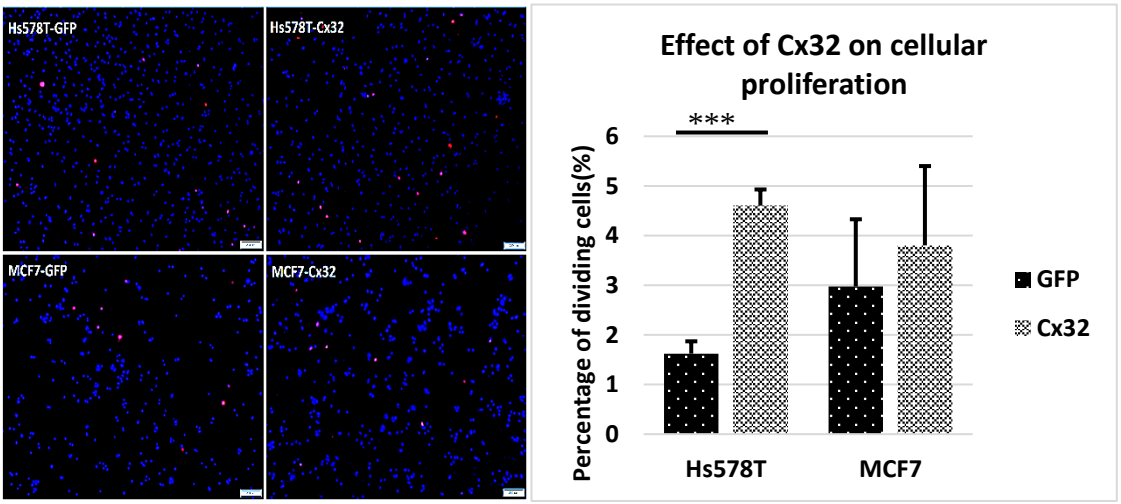

Figure 3.6. Ratio of dividing cells during two-hour period to total cells. Left panel representative images of cells taken BrdU, right panel quantification of BrdU uptake by cells Error bars represent S.D (n=3, **** $p<0.001$)

BrdU shows dividing cells directly but do not give information about cells behavior relative to confluency, while colony formation assay gives information about individual cells survival and growth potential. Similar to other experiments Hs578T Cx32 infected cells showed a significant increase in proliferation compared to control infected cells (Figure 3.7). Cx32 infection increased proliferation in MCF7 cells but that increase was not significant. Even though the change in MCF7 cells was higher than Hs578T cells (times increase in Hs578T cells), change had a wider range in MCF7 cells (no change to 4.34 times increase compared to 1.27 to 2.15 times increase).

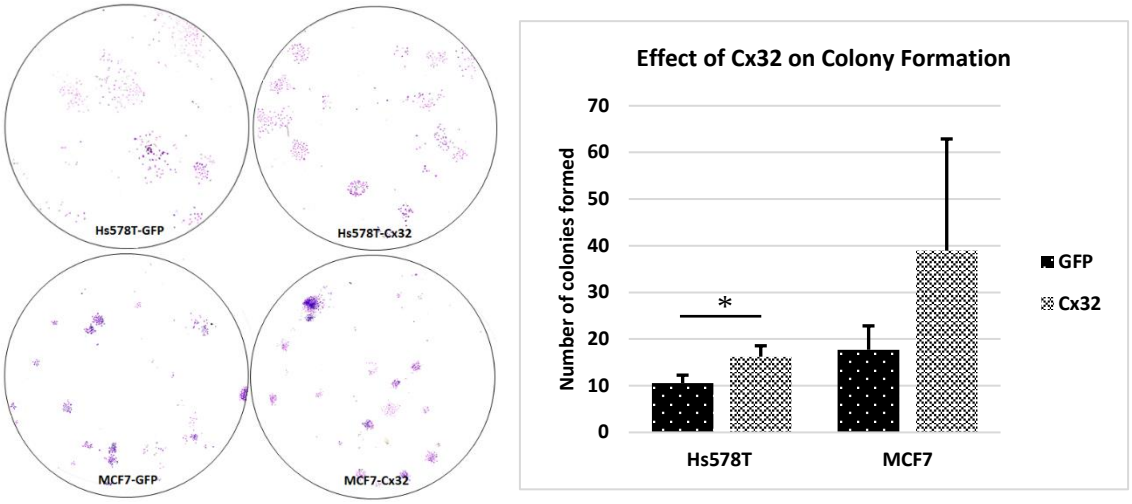

Figure 3.7. Number of colonies produced within 7 days. Left panel representative images of colonies, right panel quantification of colony formation. Error bars represent S.D (n=3, * $p<0.05$)

Connexins are long believed to be tumor suppressors due to their inhibition of proliferation of cancer cells and specially Cx43 was shown to be inhibiting growth, independent of GJIC in multiple studies (Ionta et al. 2009, Lamiche et al. 2012, Xu et al. 2008). However, their growth promoting roles were also indicated; for example, Cx43 was shown to induce proliferation in HER2(+) drug resistant SK-BR-3 and JIMT-1 cells (Yeh et al. 2017) and Cx32 was shown to increase proliferation in response to stimuli in non-myelinating Schwann cells (Freidin et al. 2009), Huh7 and Li7 hepatocellular carcinoma cells (Li et al. 2007). Most studies showing inhibition of proliferation has not been done using Cx32 or their medium was not breast cancer but Cx32 is associated with metastasis and growth in gastric, breast and lung cancers (Kanczuga-Koda et al. 2007, Jee et al. 2011, Kanczuga-Koda et al. 2010). These suggest that connexins role in cancer cells are cell type and connexin type specific, therefore increased proliferation upon overexpression of Cx32 might implicate cell or cancer type specific role of Cx32 in Hs578T cells compared to MCF7.

Connexins can alter the duration of cell cycle phases. In Rin (rat insulonama cells) Cx37 slowed down cell cycle time from 2 to 9 days, irrespective of cell density and without causing apoptosis, by delaying progression through G_1/S checkpoint (Burt et al. 2008). Similarly, in U2OS (osteosarcoma) cells, Cx43 slowed cellular proliferation by suppressing G_1/S transition without causing apoptosis (Zhang et al. 2001). Therefore, to check whether there is a difference in cell cycle progression in MCF7 and Hs578T cells

and verify the cell proliferation data, PI staining and flow cytometric analysis was performed. While infection of Cx32 did not show any effect on MCF7 cells, it significantly lowered the percentage of cells in G_1 phase (≈9%) and increased the cell percentage in S phase (≈11%) in infected Hs578T cells (Figure 3.8 and Table 3.1).

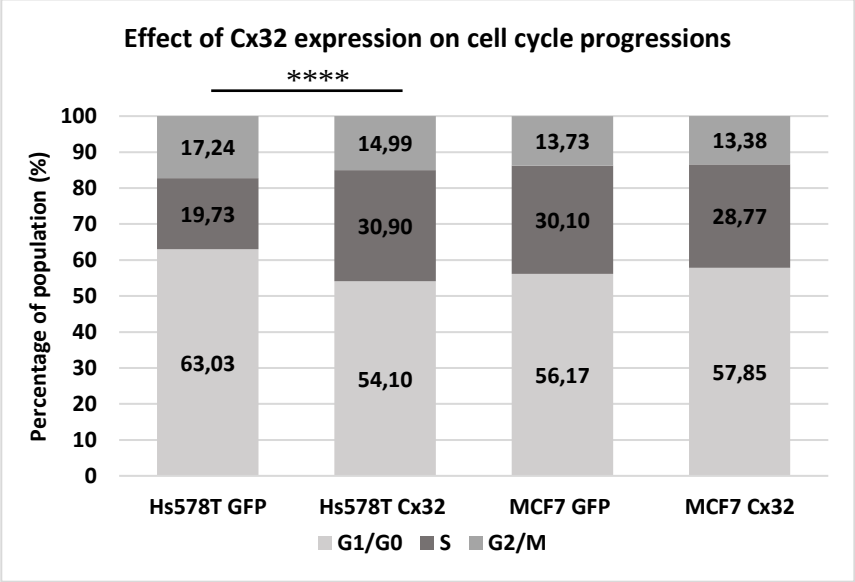

Figure 3.8. Cell cycle analysis of control infected and Cx32 infected cells.

Table 3.1. Quantification of Figure 4.14 for infected cells (n=3, **** p<0.001)

	Hs578T-GFP	Hs578T-Cx32	MCF7-GFP	MCF7-Cx32
G1/G0	63.03 ±9.28	54.10 ±5.12 (****)	56.17 ± 3.31	57.85 ± 6.67
S	19.73 ±2.50	30.90 ±3.55 (****)	30.10 ± 2.94	28.77 ± 5.11
G2/M	17.24 ±6.97	14.99 ±2.29	13.73 ± 3.94	13.38 ± 3.56

Since there was no difference at Cx32 protein levels in MCF7 cells upon Cx32 infection, observing no difference between MCF7 cells might be reasonable. As other analyses showed increased proliferation upon Cx32 infection in Hs578T cells, detecting increased percentage of cells in S phase was also expected. Generally, connexins (Cx37 in Rin cells, Cx43 in U2OS and TRMP cells) are shown to cause arrest in all phases of cell cycle through cyclins and CDKs and therefore decrease cell growth (Chen et al. 1995,

Burt et al. 2008, Zhang et al. 2001) but in our study Cx32 did not show that. One explanation for this is the fact that none of those studies were done using Cx32 or on breast cancer cells so the effect of Cx32 on breast cancer cells could be cell/tissue specific. Another explanation could be that presence of Cx32 in Hs578T cells might push the cells to act similar to MCF7 cells that endogenously express Cx32.

3.3. Effect of Cx32 on Channel Functions

Both hemichannels and gap junctions have diverse functions including regulation and signaling in various organ systems. These functions are inhibited in the presence of blockers (Kar et al. 2012). Even though hemichannels and gap junctions have similar structures and would be expected to be active in similar conditions, it is not really the case. While gap junctions communicate between two cells, hemichannels communicate between the cell interior and the extracellular environment. Therefore, their presence and activity need to be assessed individually. In order to assess the effect of Cx32 infection on the hemichannel function of cells, dye uptake assay was performed on control and Cx32 infected cells in the absence or the presence of a hemichannel and gap junction channel inhibitor CBX (Figure 3.9).

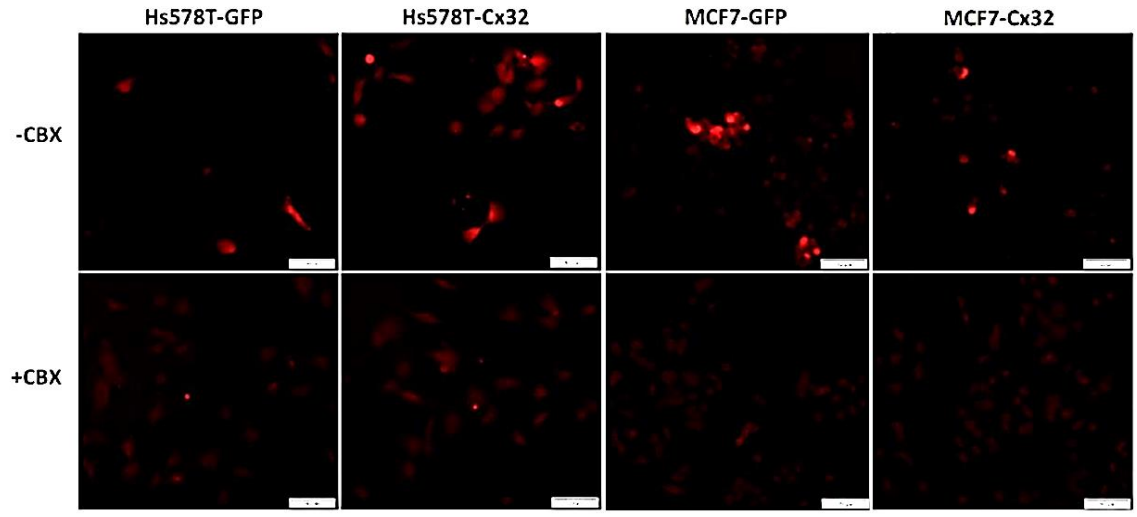

Figure 3.9. Representative images of dye uptake of control and Cx32 infected Hs578T and MCF7 cells with or without CBX. Images taken with 10x magnification (scale bar: 100μm). Red is neurobiotin.

The results showed that in general cells do not form functional hemichannels as majority of the cells did not take in neurobiotin neither in the presence or absence of CBX. For Hs578T cells it is 0.5% of the cells in the absence and 0.3% of the cells in the presence of CBX; and for MCF7 cells, on average only 0.1% of the cells took in the molecule. (Figure 3.10). This correlated to 5-10 cells per well that have functional hemichannels, meaning that for most cases, there is not many cells with open, functioning hemichannels. Still Hs578T cells had enough hemichannel functioning cells from CBX to lower, especially in non-infected cells. Since only a couple of cells had taken neurobiotin, there is no significant difference between the amount of neurobiotin taken by different cell groups (Figure 3.11). This result was expected as hemichannels are normally remain closed so they cannot take in neurobiotin. Moreover, under physiological conditions, while hemichannels open, they open very infrequently (Contreras et al. 2003). But once they open frequency of their opening does not have an impact on their function. Due to this, it is possible for overexpression of Cx32 to create a difference, but this difference was not observed.

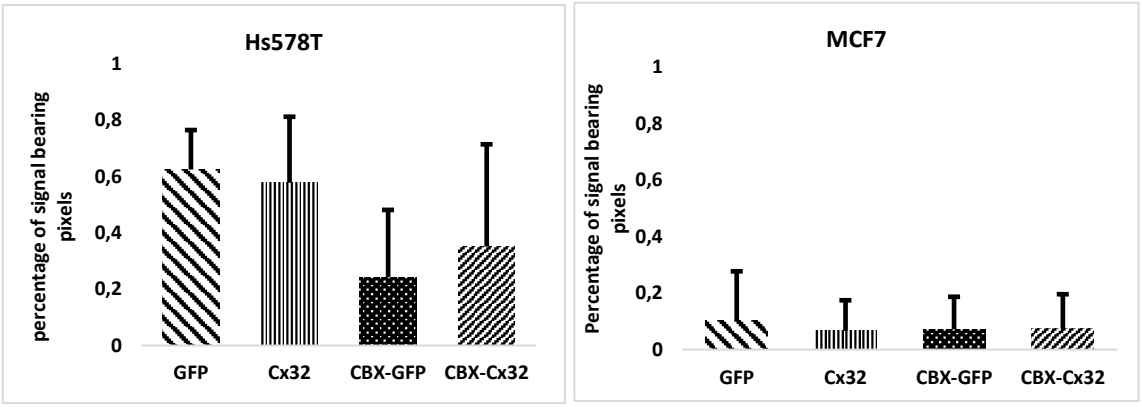

Figure 3.10. Percentage of pixels that has the signal over the threshold to all pixels with signal for all infections. Error bars represent S.D (n=3).

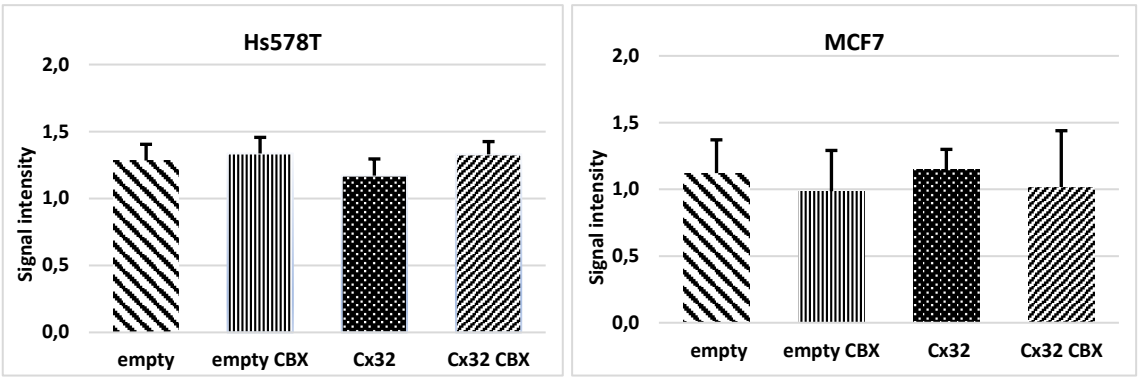

Figure 3.11. Quantification of signal strengths in dye uptake assays for all infections. Error bars represent S.D (n=3).

Even though neither cell type has much of hemichannel functionality, that does not correlate directly to gap junctional communication as communication can be independent. H161N mutation of Cx50 in oocytes for example blocks hemichannel functions while keeping gap junction activity (Beahm and Hall 2002). Therefore, in order to assess the effect of Cx32 overexpression on functionality of gap junction in cells, scrape loading assay with and without CBX was performed. Hs578T cells, formed gap junctions as neurobiotin was transferred away from the scrape line but their effectiveness significantly decreased with the overexpression of Cx32. MCF7 cells on the other hand, did not pass neurobiotin further from the scrape line even in Cx32 infected cells. This is to be expected as Hs578T cells were found to be coupling by using parachute assay while MCF7 cells did not show that level of coupling (Jiang et al. 2017). This suggests that the MCF7 cells have no functional gap junctions on the plasma membrane. In all cases, intensities of the cells that initially took up the tracer through the scrape was decreased with the introduction of CBX (Figure 3.12). According to the principle of scrape loading, the first line of cells took their tracer from the scrape line, which means CBX should not have affected the initial molecule uptake. CBX lowered communication in Hs578T cells, but since MCF7 do not pass the molecule, presence of CBX did not alter anything (Figure 3.13). Cx32 did not introduce communication to MCF7 cells, and decreased communication in Hs578T suggest that Cx32, despite being a protein that functions in communication, lowered communication rather than increase it. Considering that neither endogenous nor exogenous Cx32 in MCF7 cells directly function in communication, it is likely that introduced Cx32 in Hs578T cells directly involved in communication. Still

with the lowering of communication in Hs578T, Cx32 have an effect on present communication.

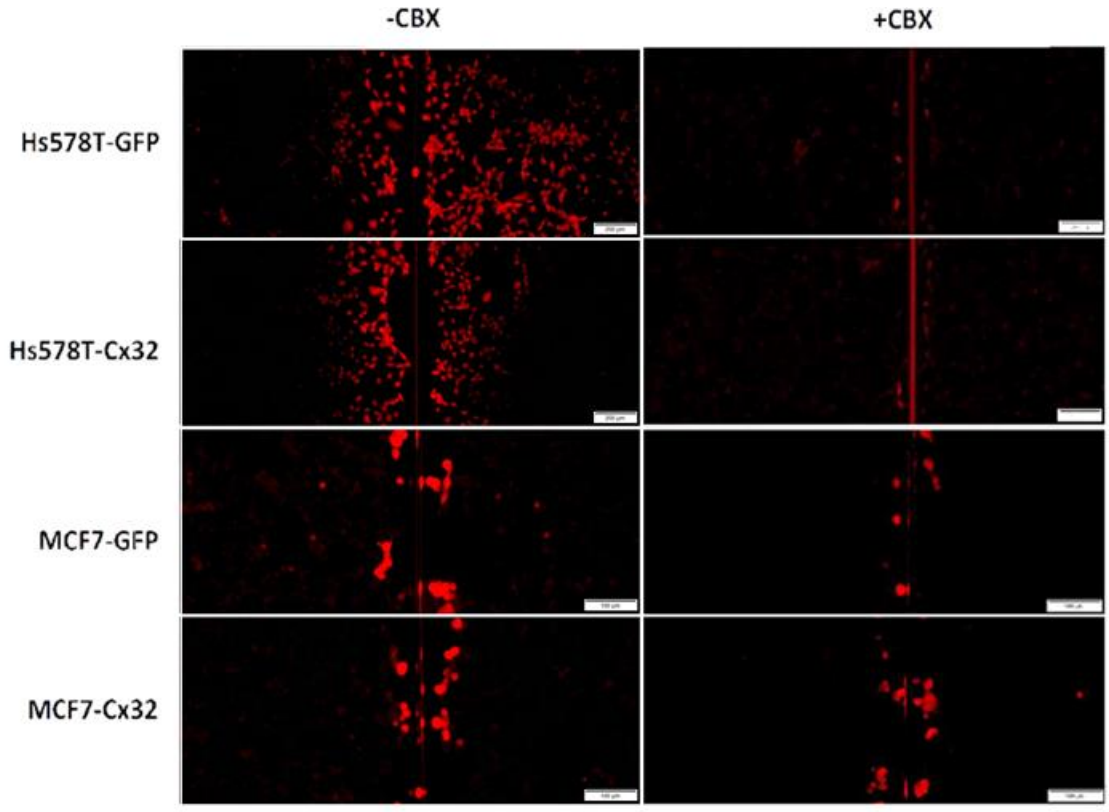

Figure 3.12. Scrape loading of control and Cx32 infected cells (scale bar: 200μm for Hs578T cells, 100μm for MCF7). Red is neurobiotin.

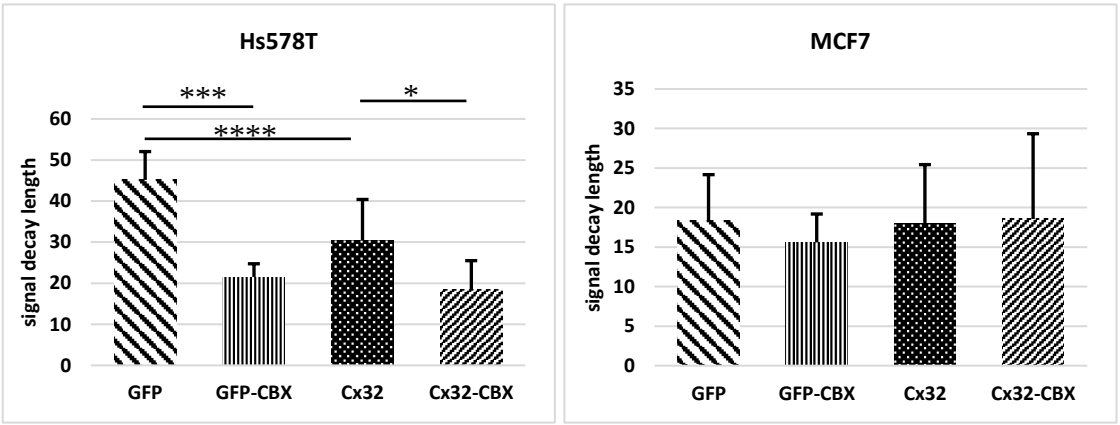

Figure 3.13. Quantification of scrape loading assays for all infections. Error bars represent S.D. (n=4; * $p<0.05$, *** $p<0.005$, **** $p<0.001$)

It can be concluded that there is not much communication for MCF7 cells as they do not seem to have functional hemichannels nor gap junctions connecting them to neither extracellular environment nor other cells. While there is more hemichannel functionality in Hs578T cells compared to MCF7 cells, their connection to the extracellular environment is still infinitesimal (0.1% to 0.5%). Despite not connecting with the extracellular environment, Hs578T cells seem to be connecting sufficiently with other cells. This communication though was lowered with the overexpression of Cx32 cells.

3.4. Intracellular Localization of Cx32 in Infected Cells

Cx32, being a gap junction protein, normally functions within GJ channels and therefore is generally found on the cellular membrane. In these breast cancer cell lines, it can either function within GJIC, or it can be moved to cytoplasm and function GJIC-independent manner similar to gastric and prostate cancers (Jee et al. 2011, Li et al. 2007). Furthermore, our dye uptake and scrape loading experiments suggested that the overexpressed Cx32 might not directly take role in GJIC. One of the possible reasons for this is that the overexpressed connexin has moved to cytoplasm. In order to determine the intracellular localization of Cx32, infected cells were immunostained against Cx32 (Figure 3.14). It was observed that, whether it is endogenously expressed (MCF7 control infected cells) or exogenously induced (Hs578T Cx32 infected cells); Cx32 was always found in the cytoplasm rather than cell-cell contact regions, as no plaque formation was observed between adjacent cells.

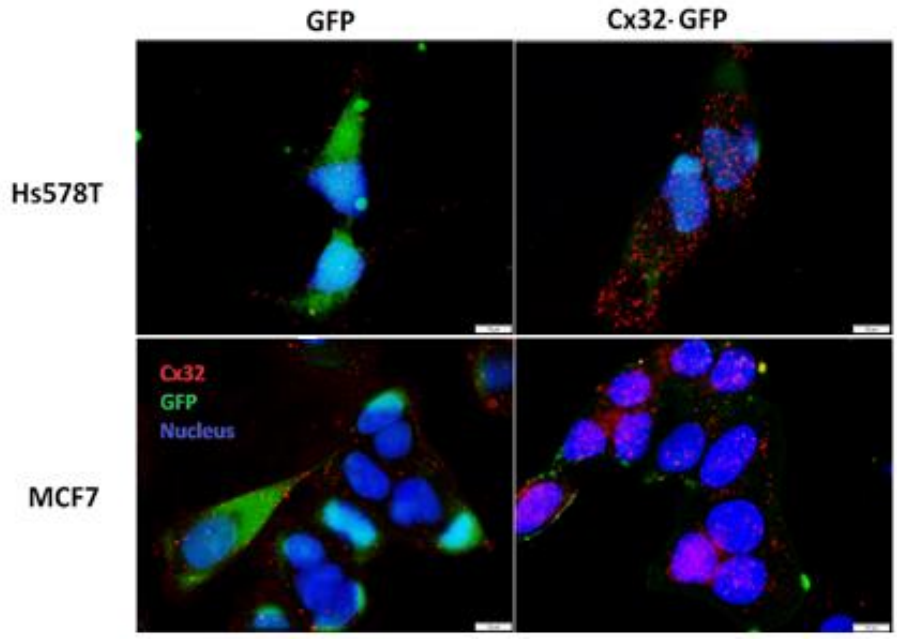

Figure 3.14. Comparison of Cx32 localization in infected MCF7 and Hs578T cells. Images taken with 100x magnification (scale bar: 10μm). Red is Cx32, Green is GFP, Blue is nucleus (images exposure has been manipulated for better visualization)

Since infected cells are green due to expressing GFP, cells needed to be transiently transfected with vectors that do not contain GFP tag to perform colocalization experiments. In order to confirm that Cx32 is not present on the membrane, cells transiently transfected with pCS2+- Cx32. Afterwards, in both untransfected and transfected cell lines, Cx32 was costained with the membrane. Similar to Cx32-only immunostaining, untransfected Hs578T cells did not show Cx32, while Cx32 transfected ones showed some. MCF7 cells showed Cx32 signal in both transfected and untransfected cells with transfected cells showed Cx32 to be more localized to unknown cytosolic sites. In both cells Cx32 gave signal in cytoplasm but not in cell-cell contact areas and shown not to be forming plaques (Figure 3.15). Even though it can be concluded that exogenous Cx32 do not form plaques, lack of plaques in uninfected (Figure 3.2) and control infected MCF7 (Figure 3.15) suggests Cx32 does not form plaques in these cells regardless of their origins.

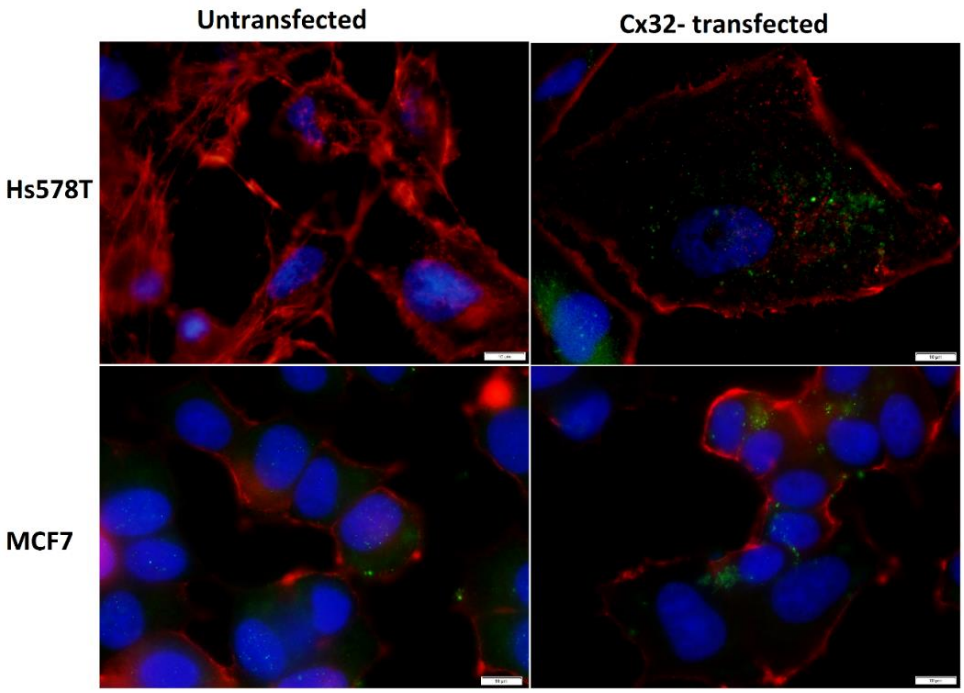

Figure 3.15. Coimmunostaining of untransfected and transfected cells. Images taken with 100x magnification (scale bar: 10μm). Red is membrane, Green is Cx32, Blue is nucleus.

Since Cx32 was not found on the membrane and were localized to the cytoplasm, next aim was to investigate its subcellular localization. Since it has been shown to be colocalized with the Golgi apparatus in prostate cancers (Li et al. 2007), we wanted to determine if Cx32 colocalizes with the Golgi apparatus in breast cancer cells as well. In order to determine this, in transiently transfected cells, Cx32 was costained with Golgi apparatus. Results showed that cells had cytoplasmic Cx32 but said Cx32 did not colocalize with the Golgi apparatus marker (Figure 3.16). This suggests either Cx32 just dispersed in the cytosol or colocalized with another organelle such as ER, in which it assembles (Rahman 1993). Cx32 localization with ER or Golgi are possible but generally when Cx32 is observed in ER or Golgi, it does not mean relocalization of the protein but retention for mutant Cx32 (Kyriakoudi et al. 2017). Since there is no indication that the exogenous Cx32 proteins in this study are mutated, it is more likely for them to be dispersed in cytosol. Since neither endogenous nor exogenous Cx32 was not found on the membrane, it possibly has functions apart from communication. Still, as Cx32 in Hs578T cells lower communication, its functions might be related with GJIC. This result is not unexpected as Cx32 was previously shown to be localized in cytosol in prostate, gastric

and colon cancers in vitro (Jee et al. 2011, Kanczuga-Koda et al. 2010, Mehta 1999) and in breast cancer in vivo (Kanczuga-Koda et al. 2007).

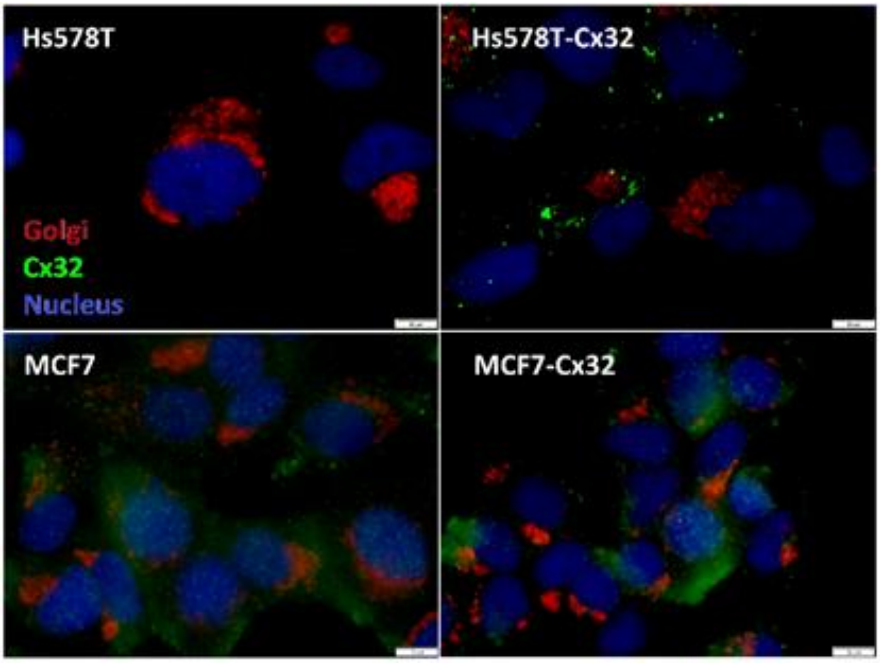

Figure 3.16. Colocalization of Golgi and Cx32 in Hs578T and MCF7 cells. Images taken with 100x magnification (scale bar: 10μm). Red is Golgi, Green is Cx32, Blue is nucleus

3.5. Effect of Cx32 on Other Connexins Within the Cell

Due to a decrease in gap junction channel permeability in Hs578T with the overexpression of Cx32, contribution of other connexins to channel functions were suspected. Since normal and tumorous breast tissue expresses both Cx26 and Cx43, their expression levels in both Hs578T and MCF7 cells were investigated. According to the literature Cx43 and Cx26 are expressed in both cell lines (Shao et al. 2005, Tan, Bianco, and Dobrovic 2002), but since expression difference in one connexin type can affect the expression of other connexins (Bedner, Steinhäuser, and Theis 2012), expressions of Cx26 and Cx43 were investigated in Cx32 infected cells for any alteration on protein level (Figure 3.17). Data showed a significant decrease in Cx43 for both Hs578T and MCF7 cells with the introduction of Cx32. Therefore, it is possible that the cause of high GJIC observed in Hs578T-GFP cells might be due to the presence of Cx43. Another

possibility for this could be the presence of another connexin that is different than Cx26 and Cx43, such as Cx46 which is known to be expressed in MCF7 cells (Banerjee et al. 2010).

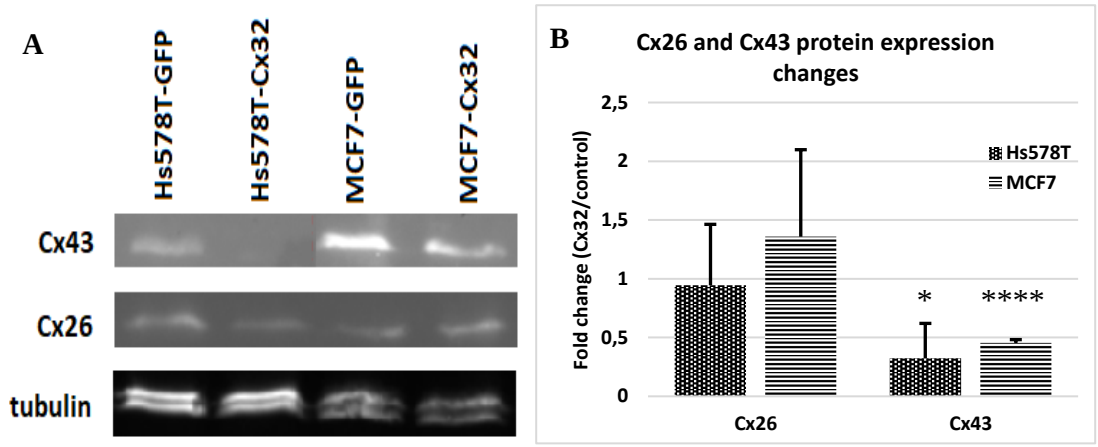

Figure 3.17. Protein levels of Cx26 and Cx43 in Hs578T and MCF7 cells A- Representative image for protein levels of Cx26 and Cx43 in Hs578T and MCF7 cells γ-tubulin was used as loading control. B- Quantification of Western blot results. Error bars represent S.D. (n=3; * $p<0.05$, **** $p<0.001$)

Through immunostaining, presence and localization of both Cx26 and Cx43 were investigated. Both connexins were present in both cell types, confirming the Western blot results. When it comes to the localization of proteins, while neither of the connexins were localized at cell-cell contact areas, Cx43 was located at cytoplasm and Cx26 accumulated at nucleus and neither connexin formed plaques (Figure 3.18 A & C). Furthermore, the connexin signal levels in labeled cells were measured. It seems that upon Cx32 infection, in Hs578T, Cx43 levels significantly decreased in signal level while Cx26 levels significantly increased. Cx26 seemed to decrease with Cx32 infection in MCF7 cells but this decrease was not significant (Figure 3.18 B &D). This data suggests that the high permeability observed in Hs578T cells dropped with the introduction of Cx32 might be due to Cx43. This is also supported by the fact that dye coupling in Hs578T cells significantly decrease with silencing of Cx43 with siRNA (Jiang et al. 2017).

The decrease of Cx43 in both Hs578T and MCF7 cells also mean that any effect observed could be directly due to Cx32 overexpression or due to the decrease of Cx43. Cx43 was shown to affect cells from cellular proliferation to migration and thus its

decrease might affect both Hs578T and MCF7 cells for other characteristics apart from channel function. On top of that, the difference might not only come from Cx26 and Cx43 as there are other connexins expressed in these cells like Cx46 which were not investigated.

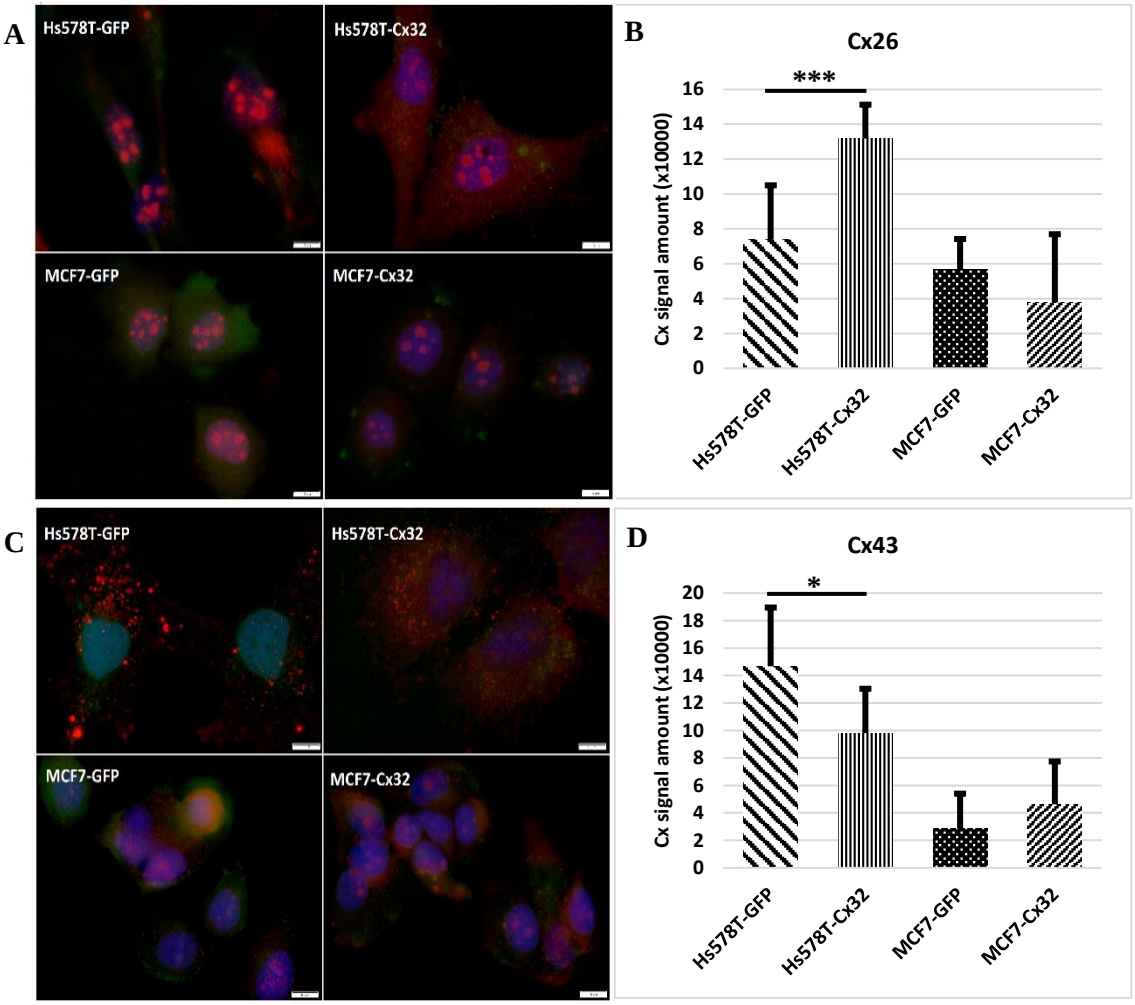

Figure 3.18. Localization and signal levels of Cx26 and Cx43 in Hs578T and MCF7 cells. A- Localization of Cx26 in infected cells. Red is Cx26, Green is GFP, Blue is Nucleus B- Quantification of Cx26 signals C- Localization of Cx43 in infected cells. Red is Cx43, Green is GFP, Blue is Nucleus D- Quantification of Cx43 signals. Error bars represent S.D. (n=3; * $p<0.05$, *** $p<0.005$)

3.6. Effect of Cx32 on Aggressiveness of Cells

Connexins are shown to be involved in adhesion and migration in both normal tissue development and in tumor progression (Kotini 2015, Sinyuk et al. 2018). Also,

Cx26, Cx32 and Cx43 were all been shown to affect cellular motility on different cancer cell lines (Cronier et al. 2009, Asadi-Khiavi et al. 2011). In order to evaluate the effect of Cx32 on migration, wound healing assay was performed. Overall, results showed that Hs578T cells have a higher migration potential than MCF7 as the open wound closed or almost closed within 18 hrs post injury in Hs578T cells. In both cell lines, Cx32 infected cell lines had significantly lower migration capacity than their control infected counterparts (Figure 3.19 and 3.20). Hs578T-Cx32 cells closed the wound 8.3% per hour while Hs578T-GFP cells closed it at 14.7% per hour. Cx32 infection reduced the wound closure to 1.8% per hour from 4% per hour.

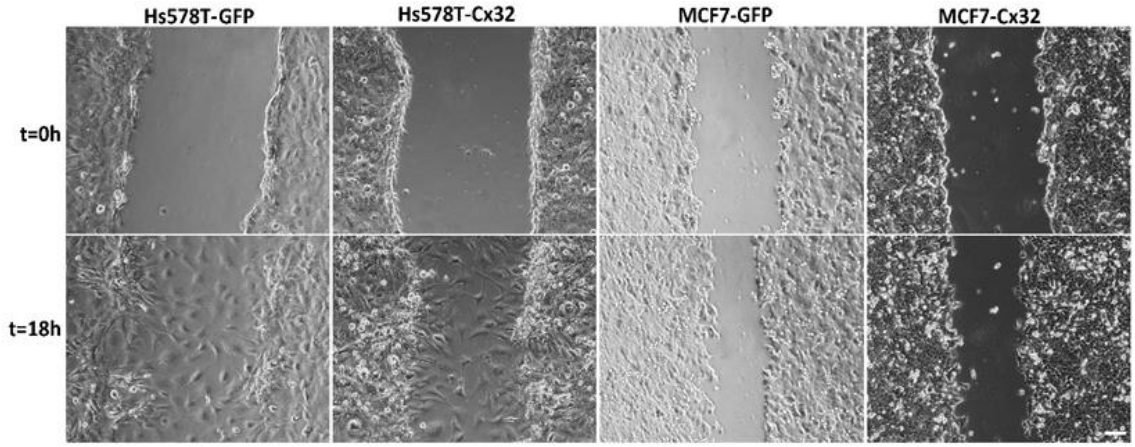

Figure 3.19. Representative images of cells taken at t=0h (right after scratch) and at 18h using time-lapse photography. Scale bar 50μm.

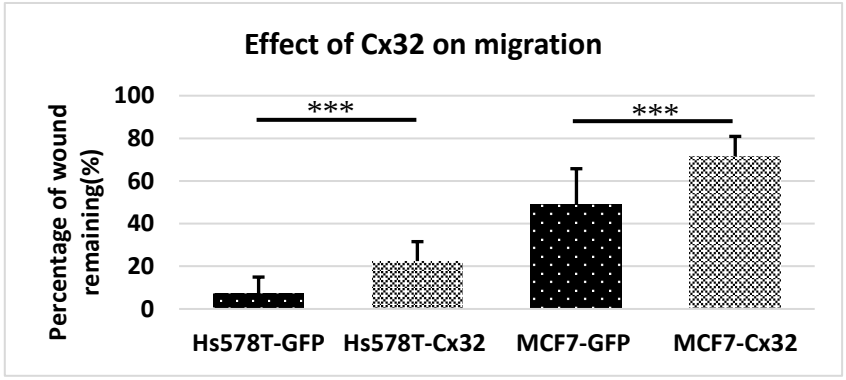

Figure 3.20. Percentage of wound remained open after 18 hrs. Error bars represent S.D (three experiments, *** $p<0.005$)

In order to assess the ability of cells to invade, an invasion assay with Matrigel to mimic extracellular matrix were used. The data showed that less Cx32 infected cells pass through the Matrigel matrix then their uninfected counterparts (Figure 3.21). For Hs578T, invaded control cells covered 20% (±1.4) of the area while invaded Cx32 infected cells covered 13% (± 1). MCF7 cells while covering less area had similar pattern, control infected cells covered 8.6% (± 2) of the area while Cx32 infected ones covered 5% (± 1) (Figure 3.22).

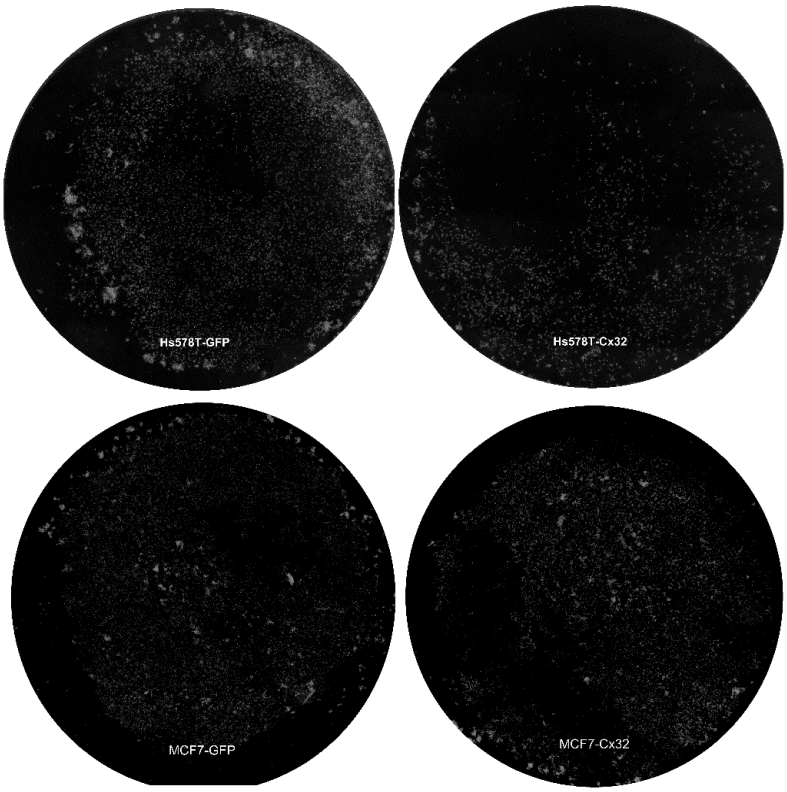

Figure 3.21. Representative images of invaded cells

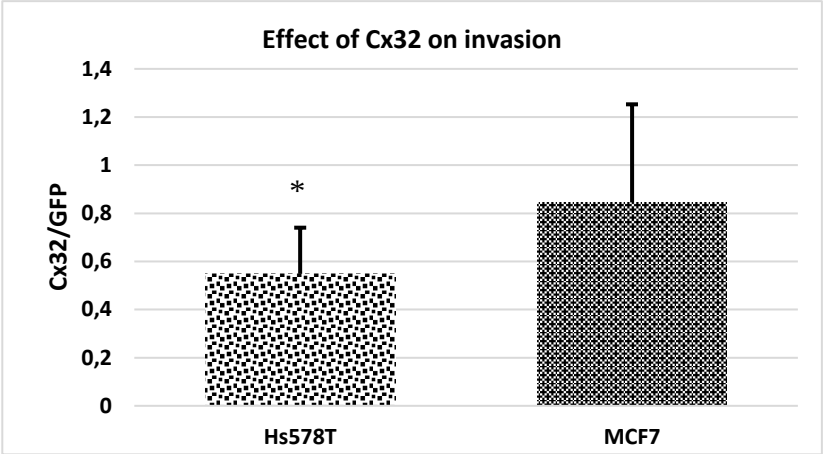

Figure 3.22. Relative area covered by invaded cells. Graph was obtained by normalizing invasion of Cx32 infected cells to control cells. Error bars represent S.D (n=3; * $p<0.05$)

The reduction of invasive capabilities with Cx32 infection is consistent with wound healing results. Since cells lose migrative ability as well as invasive ability, it can be suggested that Cx32 inhibits the cells motility, which might affect their metastasis potential. Decrease of migration and invasion with Cx32 overexpression is observed with hepatocellular carcinoma (Zhao et al. 2014) and in HeLa cells cervical cancer cells (Yang et al. 2011).

While normal cells grow attached to a surface, transformed cells can grow independent of a solid surface, therefore anchorage-independent growth is a hallmark of cancer. In order to assess anchorage independent growth, soft agar assay was performed. In soft agar assay, cells grow inside soft agar-medium mixture, laying on top of another soft agar-medium mixture. While the bottom agar prevents cells from attaching to the plate, top agar provides an environment for cells to grow without attaching any surface. Hs578T cells did not form any colonies in eight weeks in neither 30000 cells /well concentration nor 50000 cells/well concentration. In addition, Hs578T cells began degrading the agar rather than forming colonies probably by trying or achieving to move inside the agar (Figure 3.23). Contrary to Hs578T cells, MCF7 cells formed colonies within 3 weeks of seeding.

In MCF7 cells the number of colonies with radius larger than 30μm increased with Cx32 infection though the results are not significant (Figure 3.24). This result indicates that MCF7 cells gained stem cell characteristics in Cx32 infected cells. It also suggests

that either presence of Cx32 is causing this increase, which considering MCF7 cells already expressing Cx32 is unlikely; or Cx32's effect on something else is causing this increase. This suggests that this result might not be due to increase in Cx32, but reduction of Cx43 in Cx32 infected cells. In malignant melanoma cells, Cx43 overexpression suppresses anchorage independent growth (Su et al. 2000), it is also decreased by Cx43 presence in BL6 kerotinocytes (Ableser et al. 2014), in MDA-MB-231 and MDA-MB-435 breast cancer cells (McLachlan et al. 2006), and in glioblastoma cell lines U251 and T9SG (Huang et al. 1998).

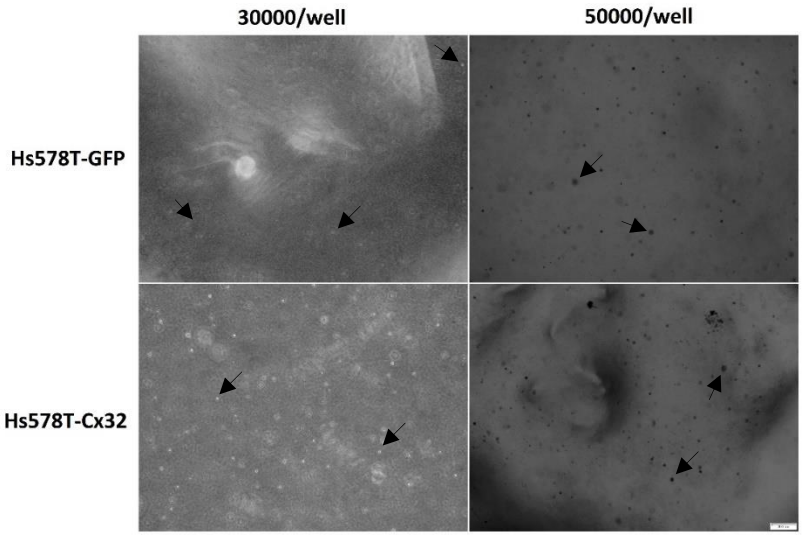

Figure 3.23. Representative images of Hs578T cells taken after 8 weeks for 30000 cells/well and 50000 cells/well concentrations. Presumed cells are shown with arrows

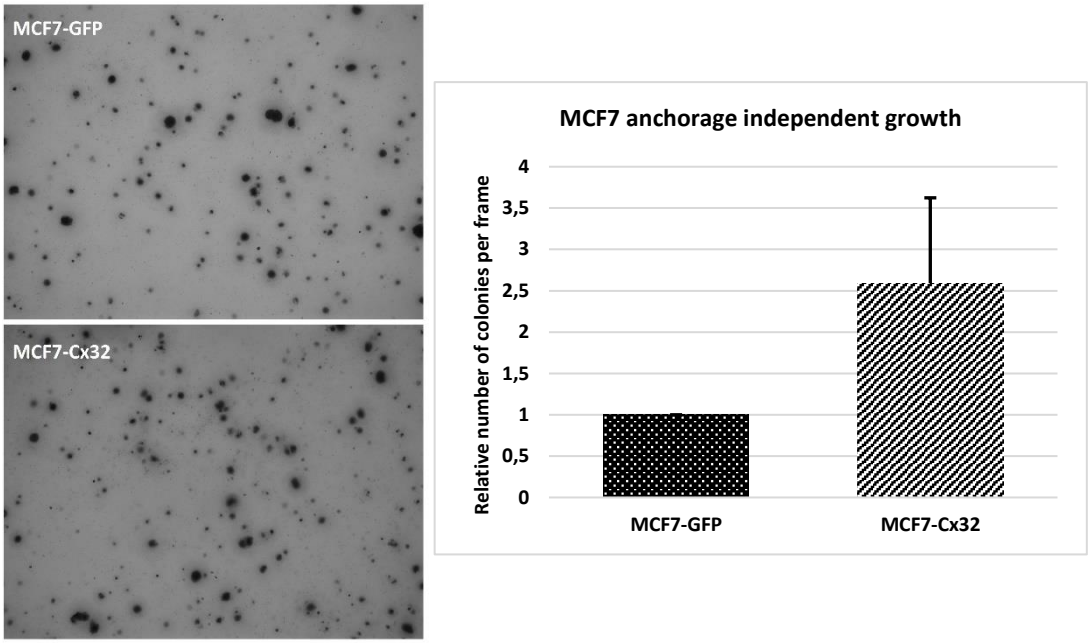

Figure 3.24. Relative number of colonies per frame. A- Representative images of anchorage independent growth of MCF7 cells. Black dots are the colonies B- Relative number of colonies. Graph was obtained by normalizing colonies produced by Cx32 infected cells to control cells. Error bars represent S.D (n=3)

One of the hallmarks of cancer is the ability of metastasis (Hanahan and Weinberg 2000) and one of the key factors in metastasis is EMT. In order to evaluate the effect of Cx32 on EMT process, changes in mRNA level of certain EMT markers were checked. The results varied notably (results not shown) thus changes in protein levels of certain EMT markers were examined with Western blot. Due to huge variations with RT-qPCR results, protein levels of the EMT markers were correlated with passage numbers. Out of all markers, only E-cadherin showed a correlation with passage number in Hs578T cells, as the passage number increased the E-cadherin difference between cells decreased (Figure 3.25). Due to having two variables (Cx32 presence and passage number), E-cadherin expression in Hs578T cells was analyzed geometrically not arithmetically.

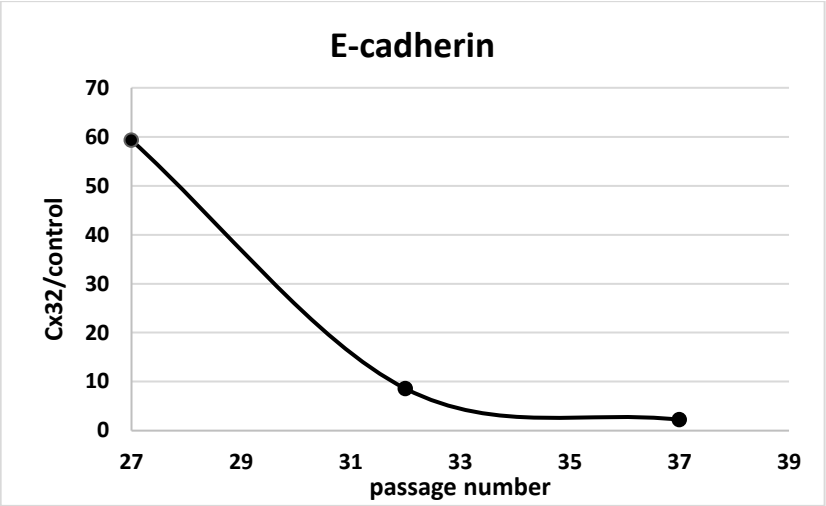

Figure 3.25. E-cadherin level change correlated to passage numbers in Hs578T cells. Graph was obtained by normalizing expression in Cx32 infected cells to control cells. Experiment performed once.

Upon analysis, it was observed that Hs578T cells showed reduction of mesenchymal and increase of epithelial marker expressions, while the data was not conclusive for MCF7 cells. Specifically, in Hs578T cells, endothelial markers E-cadherin and ZO-1 significantly increased while mesenchymal marker N-cadherin was significantly decreased. Unlike N-cadherin, other mesenchymal marker Snail also increased. In MCF7 cells, there was an increase in mesenchymal marker Slug and Zeb2, as well as epithelial marker ZO-1 upon Cx32 overexpression, but these increases are not significant (Figure 3.26). The increase of E-cadherin in Hs578T cells and increase of ZO-1 in both cells are in accordance with the decrease of migration and invasion of both cells.

A cells aggressiveness is determined by its ability to move, either within a tissue or outside of it. So, its ability to move within its bounds (migration), get free of its bounds (invasion) and move to a different location (metastasis) are all related. Cx32 overexpression in both cells decrease aggressiveness, evident from migration and invasion, as well as the expression changes in EMT markers in Hs578T cells. Cx32's effect on migration and invasion might be related with Cx43 expression in both cells but the change in EMT marker expression is possibly due to Cx32 presence as it was not observed in MCF7. Still, the general trend suggests a tumor suppressor role of Cx32 in these breast cancer cells.

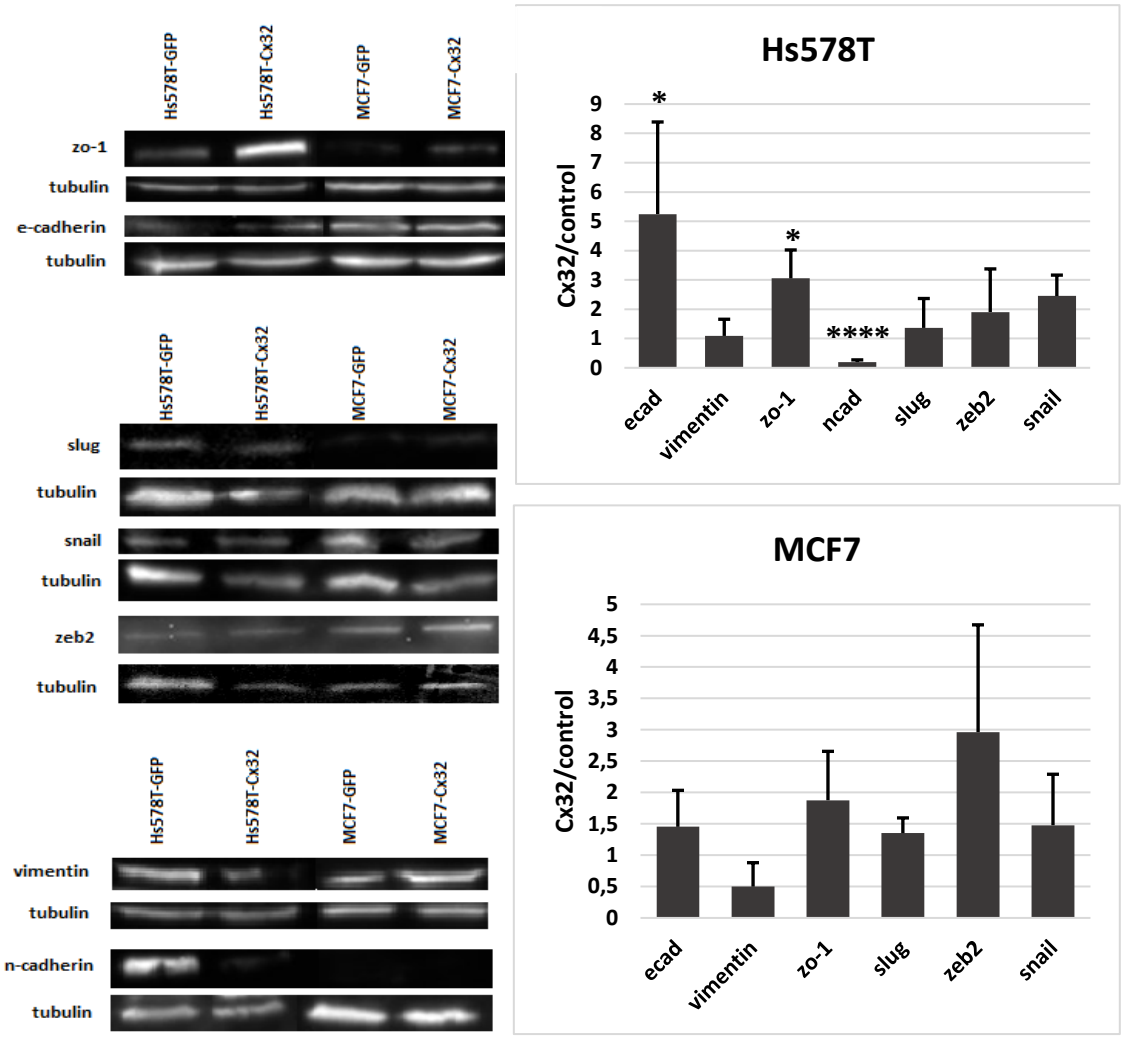

Figure 3.26. EMT marker levels in infected cells. Marker expressions were obtained by normalizing Cx32 infected cells to control cells. A-representative images of Western blot of both cell types B- quantification of data for Hs578T cells (n=3-10, * $p<0.05$, **** $p<0.001$) C- quantification of data for MCF7 cells (n=3-10)

CHAPTER 4

CONCLUSION

In this study, it was aimed to investigate the role of Cx32 on breast cancer cells with varying degrees of aggressiveness. Two breast cancer cells were used in this study, MCF7 is luminal, mildly aggressive cell line that does express Cx32 and Hs578T is moderately aggressive cell line that has been expressing negligible amounts of Cx32 (Figure 3.1). In order to observe the effect of Cx32 on these cell lines, Cx32 was overexpressed using lentiviral infection, and infected cells were assessed on multiple characteristics.

It was found that Cx32 overexpression can be achieved in Hs578T cells by lentiviral infection but total Cx32 expression in MCF7 cells did not change, probably due to the presence of endogenous Cx32. Cx32 overexpressed cells had increased proliferation in Hs578T cells possibly due to shortening of G1 phase (Table 3.1). Cx32 overexpression in Hs578T cells decreased Cx43 expression which was confirmed both by Western blot and immunostaining (Figure 3.17 and 3.18). Cx43 seems to be decreasing in MCF7 cells according to Western blot and Cx26 seems to be increasing in Hs578T according to immunostaining, but neither result is confirmed by other method. None of Cx26, Cx32 and Cx43 found to be localized at cellular membrane and thus, none of them forms plaques. Cx32 was not found to be localized in the Golgi apparatus (Figure 3.16). Cx32 infected cells also decreased in aggressiveness compared to their control infected counterparts. In both cell lines Cx32 infected cells were less migrative and less invasive (Figure 3.20 and 3.22) and in Hs578T cells Cx32 overexpression increased epithelial markers E-cadherin and ZO-1, while decreasing mesenchymal marker N-cadherin (3.26).

All of these results cannot be taken into account separately. When a factor is affected, it affects other factors as well. For example, increase in E-cadherin not only has implications for EMT but it also affects migration and invasion. Just like E-cadherin having additional roles, ZO-1 has an additional role related to channel functions. In heart ZO-1 is reported to restrict recruitment of hemichannels to cellular membrane through interacting with Cx43 (Bruce et al. 2008). ZO-1 is also found to be involved in the plaque formation for some connexins with Cx43 but subgroups other than alphas and Cx32

specially are theorized to be not involved with ZO-1 but they interact with other occludins (Hervé et al. 2007).

Apart from EMT marker proteins functioning in other processes, cells tend to show similar patterns. For example, cells generally show similar trends for invasion, migration and EMT. In hepatocellular carcinoma cell line SMMC-7721 knockdown of Cx32 enhanced invasion, migration and lower E-cadherin (Zhao et al. 2014). In HeLa cells, Cx32 inhibits migration and invasion (Yang et al. 2011). Knowing cell type specific suppressive effects of Cx32 on cancer cells, studies done on liver and cervical cancers to have similar results to breast cancer is meaningful.

Localization of Cx32 to cytoplasm from membrane is expected as this was found to be happening in other types of cancers as well. In hepatocellular carcinoma, Cx32 relocalization did not alter the total Cx32 protein expression levels but lowered Cx26 expression (Krutovskikh et al. 1995). This is similar to our findings of Cx32 overexpression lowering Cx43 in breast cancer cells.

In this study Hs578T cells and MCF7 cells showed similar patterns for most characteristics such as migration, invasion, hemichannel activity, and connexin localization but Cx32 did not have the same effect on all the characteristics that were studied. The differences could be due to MCF7 already expressing Cx32, thus the observed changes in HS578T cells might be the result of total amount of Cx32 in the cell. With the Cx32 expression rising in Hs578T cells, it became more like MCF7 cells, with lower aggressiveness, lower channel activity, higher proliferation. Even the cell cycle phases of Cx32 overexpressed Hs578T cells became similar to MCF7 cells (Figure 3.8). The changes observed in MCF7 after Cx32 infection despite total Cx32 expression not changing could be account to either the increase of Cx32 mRNA or the nature of endogenous and exogenous Cx32 to be different.

Connexin effect in cancer is generally divided into 2 groups; that are involved in GJIC and that are independent of GJIC. In this study, both are observed. MCF7 cells do not form GJIC, thus any observation of the effect of Cx32 infection happened independent of GJIC. Hs578T cells on the other hand do form GJIC of which Cx32 overexpression of decreased (Figure 3.13). Cx32 did not localize to the membrane (Figure 3.15) but without localization still lowered the GJIC that other connexins formed. This supports that Cx32 effect in Hs578T cells are not independent of GJIC.

As a conclusion, Cx32 increases proliferation, lowers GJIC, Cx43 expression, metastasis related characteristics like invasion and migration in Hs578T cells. Hs578T

with Cx32 overexpression becomes similar to faster proliferating, slower migrating, less invading, non-communicating, endogenously Cx32 expressing MCF7 cells. These results indicate a tumor-suppressive effect of Cx32 in Hs578T breast cancer cells. Though, whether this effect is directly resulted from Cx32 expression increase, Cx43 expression decrease or a combination of both is up for further exploration.

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