TIME DEPENDENT EXPRESSION AND LOCALIZATION OF CONNEXIN 32: IMPLICATION IN PROLIFERATION AND EPITHELIAL TO MESENCHYMAL TRANSITION OF MAMMARY EPITHELIAL MCF10A AND TRIPLE NEGATIVE BREAST CANCER MDA MB 231 CELLS

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ABSTRACT

TIME DEPENDENT EXPRESSION AND LOCALIZATION OF CONNEXIN 32: IMPLICATION IN PROLIFERATION AND EPITHELIAL TO MESENCHYMAL TRANSITION OF MAMMARY EPITHELIAL MCF10A AND TRIPLE NEGATIVE BREAST CANCER MDA MB 231 CELLS.

Breast cancer is the most frequent and the second leading cause of cancer-related deaths among women worldwide. Epithelial to mesenchymal transition (EMT) is critical driving force in metastasis. Connexins as a basic subunit of gap junctions indicate critical roles in regulation of EMT. In addition to Cx26 and Cx43, Cx32 is associated with breast cancer and elevated levels of Cx32 has been reported in lymph node metastasis compared to primary breast cancer while the role of Cx32 in breast cancer is still elusive. Here we aimed to shed light on the effect of Cx32 on breast cancer.

Our study suggested that Cx32 acquired mesenchymal morphology and decreased proliferation in MCF10A cells but not in MDA MB 231 cells. To further elucidate whether Cx32 indicate these changes through EMT, EMT markers were examined and subsequently it was revealed that Cx32 expression was strongly correlated with increased E-cadherin and Vimentin in MCF10A cells while decreased E-cadherin and Snail in MDA MB 231 cells. Importantly majority of Cx32 did not localize to the plasma membrane and indicated dynamic changes in a day dependent manner in both MCF10A and MDA MB 231 cells. Moreover, day dependent expression and localization of Cx32 revealed strong correlation with Zeb2 expression in MCF10A cells.

In conclusion, Cx32 indicated differential effects in regulation of EMT between MCF10A and MDA MB 231 cells. It was the first time that the role of Cx32 on EMT was investigated in breast cancer and differential localization of Cx32 was identified.

ÖZET

CONNEXİN 32'NİN ZAMANA BAĞLI İFADELENMESİ VE LOKALİZASYONU: MEMELİ EPİTEL MCF10A VE ÜÇLÜ NEGATİF MEME KANSERİ MDA MB 231 HÜCRELERİNDEKİ EPİTELYAL MEZENKİMAL GEÇİŞLERİNDE VE BÜYÜMELERİNDEKİ ANLAMI

Meme kanseri tüm dünyadaki kadınlar arasında en sık rastlanılan kanser türü olup, en çok ölümle sonuçlanan ikincil kanser türüdür. Gap junctionların en temel üyesi olan connexin proteinleri metastaz için kritik bir itici güç olan epitelyal- metastasa geçişte (EMG) önemli rol oynamaktadır. Cx26 ve Cx43 proteinlerinin yanı sıra Cx32'de meme kanseri ile ilişkilendirilmiştir. Yapılan çalışmalar doğrultusunda Cx32 ifadelenmesinin lenf düğümlerine metastaz yapmış olan meme kanseri hücrelerinde birincil meme kanseri hücrelerine oranla daha fazla olduğu gözlenmiştir. Fakat halen daha Cx32 proteinin meme kanserindeki rolü bilinmemektedir. Biz bu çalışmamamızda Cx32 meme kanserindeki etkisine ışık tutmayı amaçlamış bulunmaktayız.

Çalışmamız Cx32'nin MCF10A hücrelerinde hücrelerin çoğalmasını azalttığını ve hücrelerin mezenkimal morfoloji kazandırdığını önermiştir. Fakat bu değişim MDA MB 231 hücrelerinde gözlenmemiştir. Daha sonra, bu değişimin EMG doğrultusunda olup olmadığını ortaya çıkarmak için EMG'de rol oynayan proteinlerin Cx32 ifadelenmesi ile değişimi araştırılmıştır. Bu çalışma sonucunda görülmüştür ki Cx32 ifadelenmesi MCF10A hücrelerinde E-cadherin ve Vimentin proteinlerinin ifadelenmesiyle güçlü bir şekilde bağlantılıdır. Fakat MDA MB 231 hücrelerinde bu bağlantı E-cadherin ve Snail proteinlerinin ifadelenmesinin azalması şeklinde görülmüştür. Bu çalışmadaki diğer bir önemli nokta ise, Cx32'nin büyük bir kısmı hücre zarında sınırlanmamış ve güne bağlı olarak hücre içerisindeki yerleşiminde değişiklik göstermiştir. Ayrıca MCF10A hücrelerinde Cx32'nin güne bağlı olarak ifadelenmesi ve yerleşimi Zeb2 ifadelenmesi ile güçlü bir ilinti oluşturmaktadır.

Sonuç olarak, Cx32 EMG düzenlenmesinde MCF10A ve MDA MB 231 hücrelerinde farklı etkiler göstermektedir. Bu çalışma Cx32'nin meme kanserinde EMG' ye etkisinin araştırıldığı Cx32 'nin farklı yerleşimlerinin ifadelendiği ilk çalışmadır.

To my mother,

To the dream and hope of every child…

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CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is the second leading cause of mortality following the heart diseases worldwide. However, the ranking of mortality changes according to the geographic and demographic units over the years [1]. According to the data obtained in 2017, just in the United States, 599,108 cancer related deaths were recorded [2]. Rates of death and incidence of cancer types vary by sex and age. According to the 2017 records in the United States, in women the highest number of deaths were recorded in lung and bronchus, breast, colorectal, pancreas and ovary cancers while in men most mortal cancer types are lung and bronchus, prostate, coleractal, pancreas and liver [2]. In children, brain and leukaemia are the most common cancer types [3]. Humanity is in a war against cancer for a long time [4]. Cancer occurs as a result of many successful mutations which leads to changes in cell metabolism and functions. Chemical compounds, viruses, bacteria and radiations have clear roles in occurrence of these mutations. In addition to environmental reason, genetic mutations and epigenetic factors are other obvious players in cancer formation [3]. Unlike to its name, which is very simple and composed of a single word, cancer is not a single type of disease. Cancer is composed of many different type of disease, even between the tumours of patients having the same type of cancer indicates different molecular properties [5-7]. Cancer is a heterogenous disease and this heterogeneity leads to different patient outcomes and different treatment effectiveness [6-7]. While the day passes humanity has come to an important point in the fight against cancer where they improved screening and diagnostic techniques and advanced in therapeutic techniques. Although significant decrease in the death rate of the cancer e.g. Hodkin lymphoma, esophageal and stomach has been achieved, there is a long way to win a fight against cancer including breast cancer. Breast cancer is one of the cancer type that remained to be a global challenge especially for women, which needs innovative treatment options for late stages.[8].

1.2 Breast Cancer

Breast cancer is the most commonly diagnosed and the second leading cause of cancer related deaths in women worldwide [2, 9]. According to the data acquired from the Global Cancer Project (GLOBOCAN) in 2018, 2,088,849 new breast cancer cases were identified, and 626,679 breast cancer related deaths occurred worldwide [10-11]. When the incidence rate of breast cancer in 1980 was consider with 641,000 cases and in the light of statistic, it can be clearly seen that the incidence rate of the breast cancer has increased 3.1% annually [12]. In another aspect, breast cancer incidence rate is nearly 1 in 8 women which indicates its frequency apparently and which is independent from the income level of countries [13]. Although the initiation mechanism of breast cancer is not enlightened exactly, genetic and environmental factors have been shown to be related with breast cancer risk. BRCA1, BRCA2, PTEN, PALN2, CHEK2 and ATM can be given as an example for genetic mutations in the determination of breast cancer risk while obesity, alcohol, radiation and physical inactivity can be attributed as environmental risk factors [14-15].

As it is valid for other cancer types, breast cancer is a heterogeneous disease as well and categorized into different subgroups according to their molecular and histopathological features [16]. A female breast is mostly composed of adipose tissue and comprised of nearly 12-20 lobes, each of which is composed of lobules and these lobules connect to each other by ducts which are important in carrying the milk to nipples [17]. Most of the breast cancers are arisen in that units of terminal lobular ducts [14]. The histological subcategorization of breast cancer is based on their site and divided as invasive and non-invasive or pre-invasive breast cancers [14, 18]. In non-invasive breast cancers, proliferating cancer cells do not spread to another place from where it is originated and named according to their placed area as lobular carcinoma in situ and ductal carcinoma in situ. Invasive breast cancers occur when the cancer cells split from the lobules or ducts into another place of the breast tissue. They are the most frequent subtype of breast cancer and named according to their originated place as ductal carcinoma and lobular carcinoma [18-20]. Second categorization of breast cancer depends on their intrinsic properties, which is based on their expressed genes as basal-like, claudin-low, Her2-enriched, Luminal A and Luminal B [21]. Then, in the last but not the least breast category, cancer is subcategorized according to surrogate intrinsic factors

where expression of key proteins and histology have importance. These key proteins are hormone receptors such as oestrogen (ER) and progesterone (PR); human epithelial growth factor 2 (HER2) and proliferation marker (Ki67) [14, 22]. Tumours that are not expressing these hormone and growth factor receptors are called as triple negative breast cancer (TNBC). Another type of breast cancer types are hormone receptor positives. In these cancer cells ER and PR hormone receptors express and cancer cells use these hormones to grow. Hormone receptor positive cancer types subcategorized into two different subgroups as Luminal A and Luminal B. Luminal A group type indicates strong hormone receptor expression while shows low proliferation and also they are negative for HER2 expression [22-23]. Luminal A group cancer types accounts for 60-70% of breast cancers and have good prognosis [14, 16, 23]. On the other hand, Luminal B groups indicates lower level of hormone receptor expression and they are subcategorized according to their HER2 expression as Luminal B/HER2- and Luminal B/HER2+. Luminal B/HER2- and Luminal B/HER2+ type of cancers accounts for 10-20% and 13-15% of breast cancers, respectively and they indicate intermediate prognosis [9, 14]. HER2-enriched cancer types indicate higher human epithelial growth factor 2 and Ki67 expression while they are negative for hormone receptor expression. Among these subgroups, TNBC has the poorest prognosis and 10% of breast cancer cases belong to TNBC cancer type. All these classifications are important in the determination of therapy [16, 23]. As valid for other cancer types, the management of breast cancer comprises chemotherapy, radiotherapy, surgery and rapidly improved immunotherapy [23]. Despite the advances in therapies, according to the DeSantis reports still almost 500,000 people has died because of the breast cancer per year around the world and 90% of these deaths were due to metastasis [24]. Although the impressive increase has been made in molecular biology and immunology fields, our knowledge against breast cancer indicate major challenge for advancing these therapies and finding novel treatments. Especially the metastasis which is one of the primary reasons of mortality needs more deeper knowledge to make the therapy efficiency more long-lasting [16].

1.3 Metastasis

In cancer patients, large number of cancer cells intravasate into the circulation system every day and may lead to forming of new tumour in different sites of the body in a process known as metastasis [25]. Almost 30% of the breast cancer patients in early stage

encounter metastasis that inhibits the recovering of the disease [26]. Metastasis is a complicated process and composes of many stages which is initiated by separating of cancer cells from their originated sites and their intravasation into the bloodstream. During this process, they withstand blood vessels pressure and escape from the immune cells. Next, escaped ones extravasate from the blood capillaries and start to form colonization in different organs [4, 27].

Breast cancer metastasizes to different organs preferably to the liver, lungs, bones, the brain and lymph nodes [28]. 70% of the breast cancer patients indicate bone metastasis [26, 29]. The liver and the brain are other following preferred sites with 30% and 12-30% of breast patients have liver and brain metastasis, respectively [30-31]. In breast cancers, different subtypes indicate preference to specific organs to metastasize. Recent studies demonstrated that all subtype of breast cancer indicates tendency to bone metastasis especially HER2-enriched luminal B-like subtypes. Furthermore, non-luminal HER2-enriched ones indicate tendency to brain metastasis when compared to the Luminal A and Luminal B/ HER2- subtypes. Moreover, HER2 receptor enriched subtypes indicates liver metastasis more than the subtypes negative for HER2 receptor. Triple negative breast cancer patients mostly exhibit lung metastasis[32]. In accordance with these studies, multivariate analysis also showed that, HER2 enriched and Luminal HER2 enriched subtypes indicates more metastasis to the brain, the liver and lungs when compared to the Luminal HER2 negative. Triple negative subtypes both basal and other types indicate preference to lymph nodes, lungs and the brain. Unlike other breast cancer subtypes, basal-like ones display low rate of bone and the liver metastasis [26, 32].

In the light of clinical observations, distant metastasis does not happen on random processes. The dispersion of metastatic cells to the specific organ, which is called as metastasis organotropism, is shaped by the regulation and coherence of different factors [33]. Molecular properties of cancer cells, cancer subtypes, interactions between resident cells, immune cells and cancer cells are predominant factors among them [26, 34]. Independent from all of these specifications, metastases occurs within the first five years in all types of breast cancer, while TNBC patients indicate the worst prognosis by displaying metastasis within two years [9, 32].

After all, metastasis is one of the primary causes of mortality in the breast cancer. Every cue and findings about molecular mechanisms and dynamics behind that lethal

process can be a key improvement toward therapies. When we get the root of the issue, breast epithelial cell transformation is a combination of both genetic and epigenetic changes which are supported by favourable interactions in the microenvironment. During this complicated process, besides deregulation of proliferation, differentiation and cell survival mechanisms; the ability of invasion, dissemination and resistance to stress is also deregulated and modulated by Epithelial to Mesenchymal Transition (EMT)(Figure 1.1) [16, 28, 35]

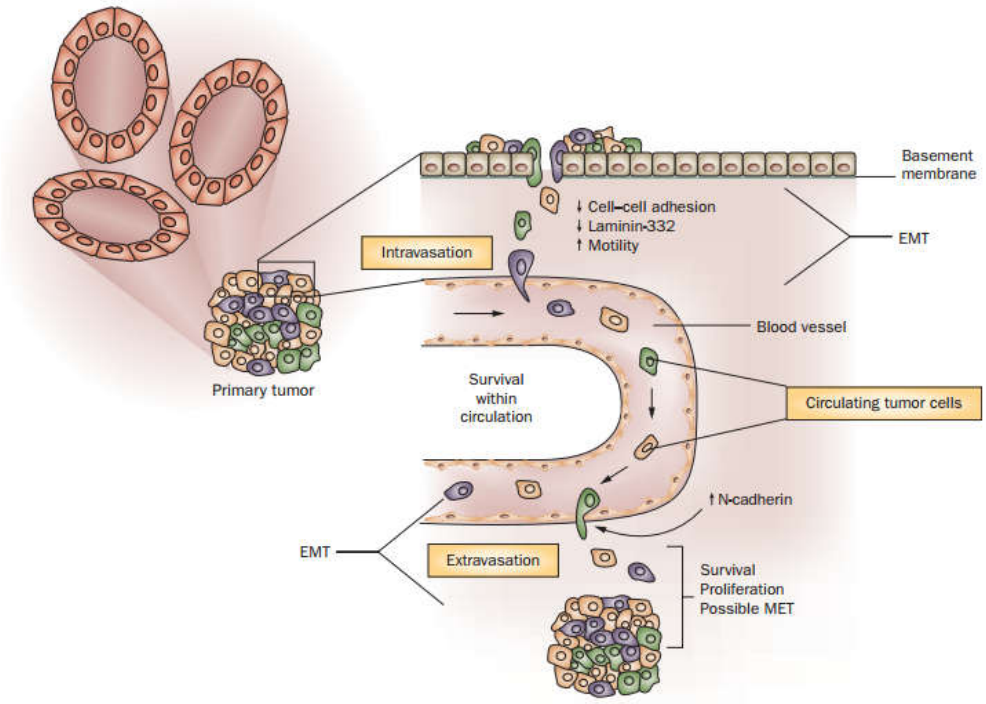


Figure 1. 1 Metastatic cascade of cancer cells accompanied by EMT.
(Source: Nauseef et al[36].)

1.4 Epithelial to Mesenchymal Transition (EMT)

Epithelial to mesenchymal transition concept was first introduced in the early1980s by Elizabeth Hay and was described as the downregulation of epithelial properties of cells and gaining mesenchymal characteristics of them during embryonic development [37-38]. In the initial times, concept was referred as epithelial to mesenchymal transformation but then, it was understood that the process was not permanent, and it can be reversed in a process of mesenchymal to epithelial transition (MET); so "transformation" word in EMT was replaced by "transition". EMT and MET is also seen

in wound healing, organ fibrosis and tissue regeneration in adults. During this process, epithelial cells progressively lose their cobblestone-like appearance with reorganization of apico-basal polarity, cytoskeletal organization and signalling programmes which are important in cell shape regulation and expression of genes required for invasive, motile phenotype (Figure 1.2). EMT provide the spindle like shape to the epithelial cells [39-41]. Besides providing phenotypes required for spreading to distant sites, EMT contributes critical phenotypes to cancer cells such as resistance to apoptosis, plasticity and immunosuppression [42-43]. According to some findings, cancer cells choose one of the two fates; they either display higher proliferation rate or display motile, invasive characters which suggest that EMT attenuates the proliferation [44-45]. During these deregulations, tumour cells can lose all of its epithelial markers and gain mesenchymal markers whereas EMT is not a process of all or nothing, instead cells can gain some mesenchymal characteristics and lose some of the epithelial characteristics which is called as partial EMT [42, 46]. Many factors are reported as candidate stimuli required for EMT promotion, such as innate and adaptive immune responses, mechanical stresses, anti-tumor drugs, hypoxia and low pH. EMT initiation stimuli can cause heritable, epigenetic changes that lead to continuation of the mesenchymal state even in the absence of the EMT stimulants. Besides environmental factors, mutations and overexpressed factors acting role in signalling pathways can also initiate the EMT [47-51].

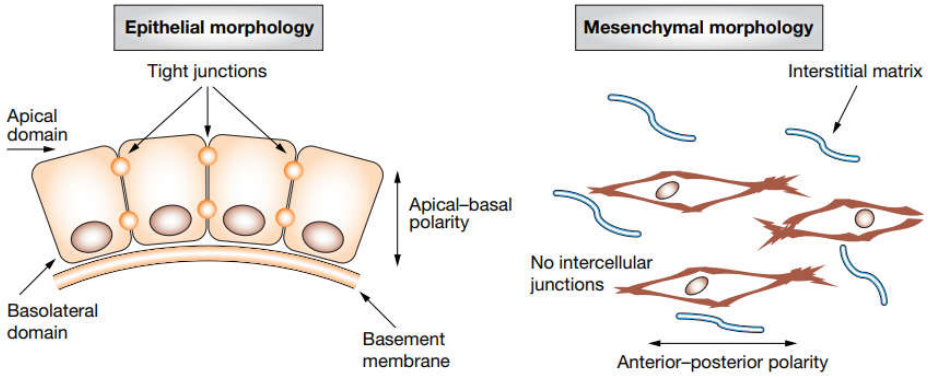


Figure 1. 2 Common epithelial and mesenchymal morphology.

(Source: Turley et al.[52])

1.4.1 Cellular Changes during EMT

Tight junctions, adherent junctions, desmosomes and gap junctions are important for holding epithelial cells together [37]. In addition, certain cytokeratins acting an important role in stabilization of desmosomes is critical in epithelial cell structure by enduring cell against different mechanical stresses [53]. Activation of EMT leads to degradation, deconstruction and re-localization of junctional proteins [37, 53]. Disintegration of tight junctions occur by decreased occludin and claudin expression and delocalization of ZO-1 from contact sites of cells [53]. EMT results in the loss of adherent junctions by the cleavage and degradation of E-cadherin. E-cadherin degradation leads to accumulation of beta-catenin and p-120 catenin in nucleus. Consequently, beta-catenin accumulation in nucleus leads to activation of target genes while p-120 accumulation leads to repression of E-cadherin expression [54-55]. Epithelial marker E-cadherin expression changes with mesenchymal cadherin marker N-cadherin [56]. In addition to changes in cadherin types, intermediate filaments change as well by decreasing some of the cytokeratin expressions and increasing vimentin expression [53]. Consequently, mesenchymal cells gain looser interaction by cadherin switching with increased N-cadherin expression and cells acquire more motile phenotype by cytokeratin switching with increased Vimentin expression which is flexible and interacts with motor proteins in the cell [53, 56].

EMT is a crucial cellular programme in malignant progression and maestros behind this orchestra are transcription factors belonging to SNAI, ZEB and TWIST family (Figure 1.3.)

1.4.2 Transcription Factors

Final result of EMT activation of signalling pathways is to activate or induce the activation of master regulators composed of SNAIL, Zinc-finger-E-box-binding (ZEB) and Twist transcription factors (TFs). EMT-TFs affect the expression of the genes cooperatively and they also influence their own expressions. All these transcription factors directly affect the EMT by directly binding to enhancer box (E-box) of E-cadherin promoter [37, 57]. Roles of transcription factors are summarized in Figure 1.3.

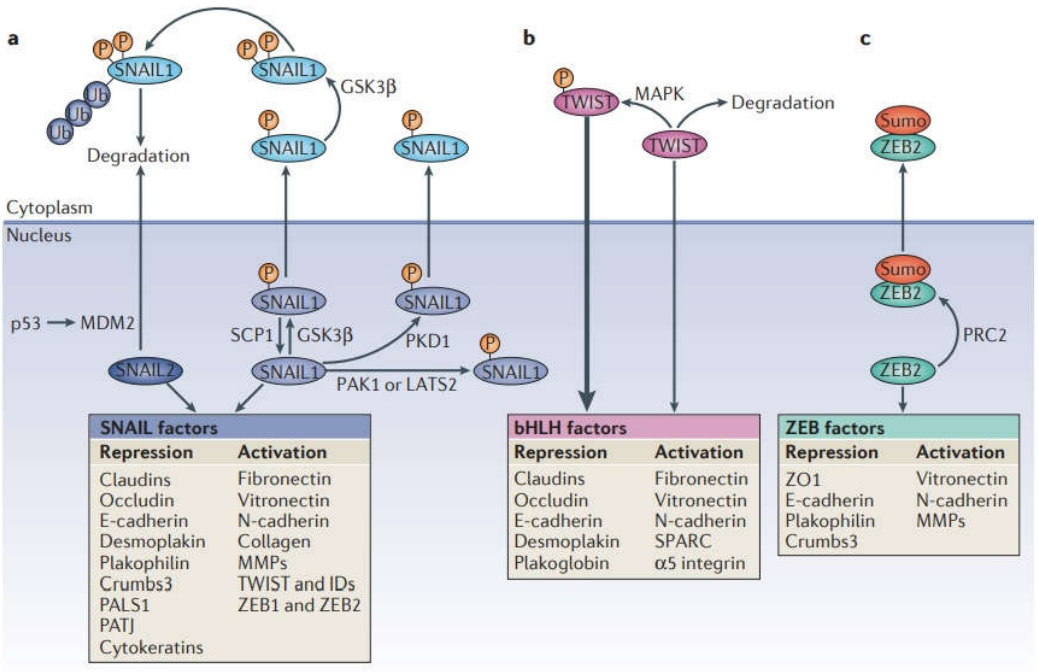


Figure 1. 3 Post-translational modifications and role of (a) SNAI (b) TWIST and (c) ZEB Family transcription factors on EMT.

(Source: Lamouille et al [37])

SNAI transcription factors/transcriptional repressors: In vertebrates three different zinc finger transcriptional factors, SNAI proteins, are found as SNAI1 (Snail), SNAI2 (Slug) and Snai3 (Smuc). Two of them namely Snail and Slug are known with their direct effect on EMT by decreasing E-cadherin expression, and also with direct or indirect effect on EMT by upregulation of mesenchymal markers expressions [58]. Snail role in metastasis is reported in a variety of cancers including lung, ovarian and breast cancers [59-60]. In 2007, it was revealed that Snail is necessary for the dissemination of MDA MB 231 breast cancer cells to lymph nodes and in accordance with this finding downregulation of Snail by silencing RNA leads to decreased metastasis and suppression in immune system [61]. According to the findings based on murine models, besides Snail effect on aggressiveness of the cancer cells, Snail expression is associated with immunotherapy resistance in breast and melanoma carcinomas [59, 62]. In addition to its effect on EMT induction, its role in cell cycle regulation and apoptosis is described with several reports as well [44]. Slug which is another SNAI family member known as a crucial transcription factor in neural crest development is important in metastasis of melanoma cells. Besides melanoma, Slug expression increases in lung, colon and ovarian cancers as well [63]. Findings revealed that

basal-like breast carcinoma also displays high Slug expression and Slug is also important with its assistance to Twist as an indication of Twist's role on EMT [37, 63]. Snail and Slug promote chemoresistance by counteracting the apoptosis mediated by p53 and by modulating genes acting in maintenance of the stem cells and cell death [64].

SNAI family is important that most of the time as an initial step Snail expression is activated in the induction or advancement of EMT [37]. In addition to Zeb family and Twist transcription factors, Snail also indicates its effect along with other transcription factors such as Snail interacts with SMAD family to display TGFB effect on E-cadherin repression [65]. Notch, Nf-κB, FGF, EGF, TGFB-SMAD3, Wnt-beta-catenin signalling pathways activate the Snail and Slug expression [57]. In addition to these, Snail also represses its own expression with an autoregulatory loop and Slug can activate its own activation after the induction of BMP-SOX2-PKA pathway. However, the latter function is surprising for SNAI family which are commonly known as a transcriptional repressor [66].

Besides their expression, these signalling pathways are also important in their post translational modifications which is important in their localization, degradation and activation. Phosphorylation of Snail in Ser97 and Ser101 motif leads to nuclear localization while following phosphorylation in the second motifs at Ser108, 102, 116 and 120 leads to ubiquitin dependent degradation. Wnt and PI3K-AKT pathways leads to Snail activation by inhibiting GSK3beta dependent phosphorylation of Snail [67-68]. Some specific phosphatases such as SCP induces its nuclear export as well to promote EMT. MDM2 interaction of Slug also facilitates the recruitment of the Slug to tumor suppressor p53 for degradation [69] (Figure 1.3 (a)).

ZEB transcription factors: Zeb1 and Zeb2 can act as both activator and repressor by binding to E-boxes of genes and they are key factors in E-cadherin repression and associated with malignancy in different cancers [70]. ZEB family proteins indicate their effect with their SMAD interaction, C terminal binding and p-300-p/CAF binding domains. ZEB and Snail reciprocally regulates each other's expressions [71]. Findings revealed that Zeb1 enhances the proliferation by decreasing the p21 expression [72] and Zeb1 is associated with poor differentiation and invasion depth in breast cancer cells and in gastric carcinoma expression. Besides gastric cancer, Zeb1 is associated with poor prognosis in colorectal, gastric, pancreatic, ovarian cancers and hepatocellular,

oesophageal squamous carcinoma [73]. Zeb2 is another member of zing finger E-box binding protein family and in addition to E-cadherin, Zeb2 directly represses the desmosomal and gap junctional gene expressions. Besides, in bladder cancer it is demonstrated that in aberrant Zeb2 expression, cells are protected from apoptosis induced by DNA damage which is very important in cancer progression. According to clinical data, as with Zeb1, Zeb2 expression is associated with poor prognosis, differentiation and metastasis of tumour and it is associated with poor prognosis in ovarian, gastric, bladder and pancreatic cancers [74].

As seen in SNAI, ZEB proteins expressions are induced by some proteins acting role in signalling pathways such as TGF-β, WNT and RAS-MAPK. microRNAs and sumoylation attenuates the expression and activation of ZEB proteins, respectively (Figure 1.3. (c)) [37].

Twist transcription factors: TWIST belongs to the helix loop helix transcription factors which are important in specifications and differentiations of lineages [75]. As with transcription factors described above, TWIST increases the mesenchymal gene expressions while downregulates the epithelial gene expressions. Independent from SNAI transcription factors, TWIST increases the N-cadherin expression while decreases the E-cadherin expression. Variety of signalling pathways regulate TWIST expression in both embryonic development and cancer progression [37, 76]. In cancer, remarkably hypoxic conditions, hypoxia inducible factor induces the TWIST expression. Besides, mechanical stress is also revealed as an inducing factor in TWIST expression with researches performed in Drosophila melanogaster [70]. As with SNAI, TWIST stabilization also depends on the phosphorylation at certain sites to protect it from degradation with ubiquitin dependent mechanism and to increase its activity (Figure 1.3. (b)) [77].

Taken together, EMT is regulated both by intrinsic and extrinsic factors and during this critical cellular process, proteome and signalling pathways are regulated elaborately. Cell to cell communication is among these driving forces in altering signalling pathways and for more than 50 years the role of gap junctions in cancer which provides the intercellular communication has been studied which has recently started to translate into the clinics.

1.5 Gap Junctions

The discovery and identification of the gap junctions role and importance in cancer progression has been studied for more than 50 years and its complicated, stage and context dependent role draw attentions to that topic [78-79].

Gap junctions come into existence with specific intercellular aqueous channels and bridge the two adjacent cells directly from their cytoplasms and permits the transmission of the secondary messengers and small metabolites smaller than 1 kDa including cAMP, ATP, Ca^{2+} and microRNAs (Figure 1.4 (e)) [80]. The interchange of both short- and long-range signals between cells are referred as gap junctional intercellular communication (GJIC) and is critical in important processes in the cell including proliferation, differentiation, apoptosis and in tissue homeostasis [81-82]. The name 'gap' is derived from the occurred 2-3 nm gap between the cells connected by these channels. These intercellular channel arrays are formed by connexin family in chordates [83].

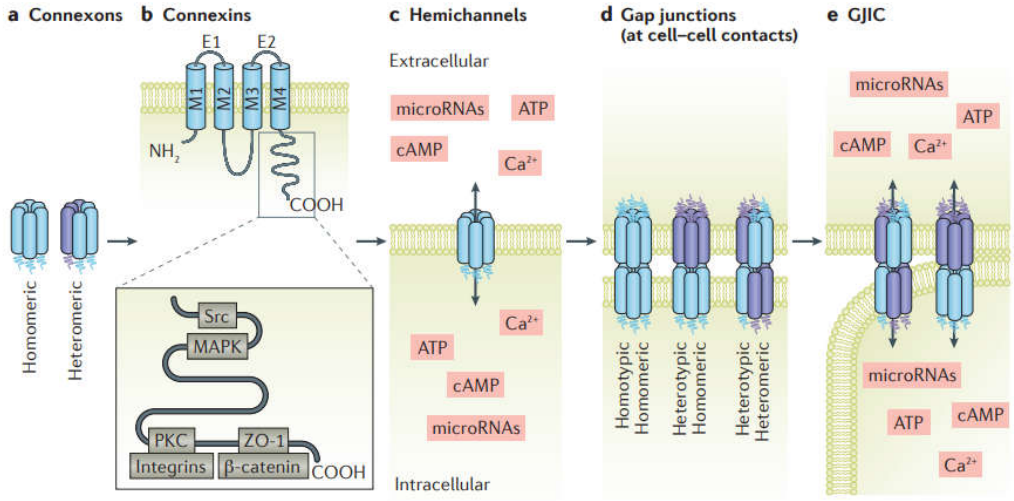


Figure 1. 4 Representative image of (a) connexons, (b) connexins, (c) hemichannels, (d) gap junctions and (e) GJIC

(Source: Donehue et al[84])

Connexin proteins are comprised of two extracellular loops, one cytoplasmic loop where variations mostly occur and having docking sites for many signalling proteins and four alpha helical transmembrane domains (Figure 1.4 (b))[84]. The range of their molecular weight is between 26 kDa and 57 kDa. Connexin proteins are named according to their

molecular weight as Cx26 and Cx57 [85]. Another nomenclature is based on the Greek letter based on subfamily that they belong to and the number in which order they are discovered. Connexin family is divided into alpha, beta, gamma, delta and epsilon subfamilies according to similarity in their sequences [86]. Mostly connexins interact with a connexin found in the same subfamily during channel formation [25, 78].

In human, 21 different connexin isoforms are found and their expression occur in tissue specific manner. Connexin found in every tissue of our body and more than one subtype of connexin is also seen in most of the cell types [78, 87]. The clearest sample about this is based on the connexin distribution in cardiac cells. Cx43 expression is seen in almost all the cardiac parts except sinoatrial (SA) and atrioventricular (AV) nodes while Cx40 expression is limited to AV nodes and His-Purkinje cells. Besides Cx40, Cx45 is expressed in AV and SA nodes as well [88]. Lastly, Cx43 is the most extensive connexin isoforms found in most of the tissues [78-79].

Six connexin members oligomerize and form a hemichannel which are called as connexons (Figure 1.4 (a,c)). Formed hemichannels then assembled with another hexamer from a neighbouring cell and forms a gap junction on the plasma membrane. Connexon formation can occur both in endoplasmic reticulum and in trans-Golgi network depending on the type of connexin isoform [78]. For instance, while Cx43 assembles into connexions in the trans-Golgi network, Cx26 and Cx32 assemble into their connexons in the endoplasmic reticulum. Then they are transported to the plasma membrane. As with other membrane channel proteins, hemichannels and gap junctions are open and close according to the presence of the stimuli [89-90]. Calcium concentration and pH are among these stimuli and they permit the transmission of the specific molecule, meaning that each connexin unit indicates selective permeability to different substances [91]. For instance, while Cx32 and Cx43 is more permeable to the anionic substances, Cx45 is more permeable to the cationic substances [92].

As mentioned above, more than one type of connexin can be expressed in the same cell and that's why connexons can be either homomeric as formed by oligomerization of the same type of connexins or heteromeric when comprised by different types of connexin subunits [93]. In addition to connexons, gap junction channels can be homotypic and heterotypic based on their connexon composition as well (Figure 1.4 (d)) which may indicate the hybrid or one connexon characteristics in preference of

substance. In here it should be noted that not all of the combination of connexins form a functional connexons, hemichannels for example in prostate epithelium Cx32 and Cx43 found in different type of cells as luminal and basal, respectively and the connexon formed from oligomerization of these two connexin type do not form functional channel [94].

As with transcriptional factors, post translational modifications also affect the activity, stability and localization of the connexins. Phosphorylation is a very largely observed one and is a key regulator in hemichannels and gap junctions as well as in connexons regulation. Phosphorylation acts as a molecular switch in channels and a regulator in non-canonical functions of connexins [95]. S-nitrosylation is another post translational modification which is arisen with covalent binding of NO to cysteine residues. In Cx43, S-nitrosylation occurs in C terminus and affect the permeability of the channels. Besides Cx43, nitrosylation is observed in Cx26 and Cx37 as well. Sumoylation, acetylation and ubiquitination are other post translational modifications, playing role in connexon and gap junction regulations [95].

1.6 Non-Canonical Functions of Connexins

Besides their canonical functions as in gap junction plaques, connexins can display non-canonical functions as hemichannels and signalling hubs where they can interact with other proteins acting a role in signalling pathways. In signalling roles, cytoplasmic tails of connexin isoforms act as a specific docking platform to specific proteins [78].

Until to date, many researches revealed the connexins interactions with proteins acting in cell death and proliferation. Cx43 interaction with beta-catenin is revealed in several reports [96-98]. According to the report presented in 2000, overexpression of Cx43 leads to the sequestration of beta-catenin to the plasma membrane as shown by the aid of reporter genes in cardiac myocytes [99]. Sequestration of beta-catenin in the plasma membrane prevents its translocation into the nucleus which is important in expression of beta-catenin's own expression and expression of genes acting role in proper cardiomyocytes function. In a research performed in 2013 [100], it was suggested that Cx43 indicates its antitumor activity by sequestering beta-catenin and do not allow its translocation into the nucleus in MCF-7 and MDA MB 231 cells in 3D culture which is

proved by immunostaining experiments. In 2017, beta-catenin-Cx43 interaction was reported in osteoblast cells which results in decreased cell proliferation. Besides beta-catenin, opposite interactions between different cadherins and connexins are reported by several reports [97]. Govindarajan and his colleagues showed the positive and negative regulation of E-cadherin and N-cadherin on GJ assembly, respectively [101]. In 1997 Fujimato et al. showed the colocalization of E-cadherin with Cx26 and Cx32 in mouse liver cells which attract to focus of researches to that issue [102] and decrease in proliferation was shown with an E-cadherin increase mediated through Cx43 overexpression in researches. In another aspect when concerning the interactions between cadherin and catenins that they interact with each other in the cell (exp E-cadherin and beta-catenin); the interrelation between connexins and these two proteins are inevitable. Recent findings revealed the colocalization between connexins with ZONAB (Zonula occludens 1 associated nucleic acid binding protein) and ZO-1 proteins [103-104]. Cell cycle dependent interaction between ZO-1 and ZO-2 proteins with Cx43 is demonstrated clearly. In this research in the light of immunoprecipitation and MS/MS analysis it was revealed that Cx43 indicates preference to interact with ZO-1 in G1 phase while it indicates preference to ZO-2 in S phase of cell cycle. However, the role of their interaction is elusive [105]. Until to here most of the samples given for non-canonical function of connexins are mostly related with Cx43 which has a long tail comprises many different tales. In addition to these adherent junctions, connexins indicate interactions with tight junctions with different isoform such as Cx32. It is known that Cx32 interacts with Disc large homolog 1 (Dlgh1) protein which belong to the tight junction family. Duffy and colleagues demonstrated that Cx32 is responsible for expression of Dlgh1 which translocates to the nuclei then enhances the cell proliferation [106]. Besides Cx32, Cx43 and Dlgh1 interaction has also been shown in the cytoplasm of transformed cervical cells but not in non-transformed cervical cells suggesting a role in transformation of tumour cells [107]. Besides, connexins interaction with apoptotic proteins are also known. In human breast cancer, cytoplasmic colocalization has been reported between Bak protein with Cx26 and Cx43 proteins. Giardina and colleagues also presented the interaction between Cx43 and apoptosis signal regulating kinase 1 (ASK1) protein [108].

All of these examples demonstrate only some of the non-canonical functions of connexins, still there are many pathways remain to wait enlightened that connexins take roles. It is clearly seen that connexins do not have so simple and straightforward

functions. Another striking non-canonical function of connexins is based on their effect on gene regulation. According to the researches it has been demonstrated that connexins can directly affect the transcription. Recently in 2018, Kotini and collegues reported Cx43 as a direct transcription regulator for N-cadherin expression [109]. In addition, Dang and colleagues revealed translocation of C terminal portion of Cx43 into the nucleus accompanied with decreased proliferation in Hela cells and cardiomyocytes [110].

1.7 Mammary Gland and Connexin Expression

In human mammary gland Cx26 and Cx43 are the most abundantly expressed connexin isoforms. In addition to these Cx30 and Cx32 are the other reported connexin isoforms in mammary gland. As mentioned above, mammary gland is composed of many lobules producing milk and connected to nipples by the aid of ducts [111]. Localization of connexin isoforms in mammary gland differs from each other. Cx26 and Cx32 are localized in luminal epithelial cells, while in myoepithelial cells Cx43 is the only detected isoform [112-113]. Mammary gland development is regulated by many different signalling pathways from birth to pregnancy. So, the differential expression of connexins during different stages is not surprising. The exploration of the connexin isoforms in different stages of differentiation is based on the tissue samples taken from mouse mammary gland due to the difficulties taking the tissues from human mammary gland [112]. Based on these researches, in the light of microarray studies Cx26 which is the first identified connexin isoform in mammary gland is revealed in all stages from virginity to the involution while its expression expanded to pregnancy and display peak in lactation. All these findings suggest its role in homeostasis and differentiation of mammary gland [112, 114]. Cx30 and Cx32 isoforms are detected in lactation state. In addition to these, Cx30 expression is detected in pregnancy and declines in involution while Cx32 expression is detected in parturition. As with Cx26 and Cx32, Cx30 also localized in luminal cells [115-116]. While in initial studies Cx30 and Cx32 were not reported in human mammary gland, advanced researches identified them in human mammary gland. In 2006, it was reported that Cx30 forms a hemichannel with Cx26 in human mammary gland which then suggested a role of Cx30 in regulation of milk production [115]. Besides, in 2014 Teleki and colleagues identified Cx32 between luminal epithelial cells for the first time. In the same research it was detected that Cx32 is associated with a favourable overall survival rate in ER positive Luminal B group breast cancer patients[117]. Unlike to Cx30, Cx32 and Cx26; Cx43

disappeared in lactation and decreased in mid-pregnancy and re-expressed in involution. As mentioned above Cx43 localizes in myoepithelial cells and all these findings suggest that it has a role in differentiation and growth of myoepithelial cells [112].

1.8 Connexins and Breast Cancer

Great deal of researches proposes connexins role in cancer pathogenesis pointing out their both pro-tumorigenic and anti-tumorigenic roles. In the light of initial clinical researches connexins are considered as tumour suppressors with evidences about the loss of gap junctions in primary tumours. Then, several knockout mice models of different connexins also supported these reports. However, advanced research remarks connexins complicated functions due to their stage and context dependent expressions and functions [79, 118]. Cx26 and Cx43 which are widely expressed connexin isoforms in mammary gland are the most studied isoforms in breast cancer. In the light of molecular analysis different function of connexins in cancer pathogenesis is identified in both anti-tumorigenic and pro-tumorigenic manner. Cx26 and Cx43 indicates barely cancer subtype dependent effect and expression especially for Cx43 in breast cancer [117]. As mentioned above Cx43 expression changes throughout the development of mammary gland suggesting the effects of hormone on Cx43 expressions. So it is not surprising that Cx43 expression indicates differences between different subtypes of breast cancers. According to the mRNA analysis of clinical samples, Cx43 expression is high in ER and PR positive subtypes. Further, based on the gene array analysis, Cx43 expression is associated with a better prognosis in ER positive subtypes while indicates poor prognosis in HER2 positive and ER negative subtypes [119]. Moreover, Elzarrad and colleagues reported the increased lung metastasis from pooled breast cancer samples due to the overexpression of Cx43 [120]. In addition to Cx43, Cx26 is also used as a prognostic marker in breast cancer as many clinical researches revealed the strong association between Cx26 expression and poor prognosis. Naoi and colleagues also supported with a report based on clinical samples by indicating association between Cx26 expression and lymph node invasion, tumours growth and poor prognosis [121].

In accordance with these clinical findings which comprises the samples from primary tumours to the sections taken from invasive and metastatic one's; researches based on breast cancer cells indicated their different roles and functions as well.

According to the organoid research, Cx43 overexpression indicates gap junction intercellular communication independent mechanism and leads to decrease in cell migration and increase the mesenchymal to epithelial transition[122]. In addition to these, in the same research it was revealed that Cx26 and Cx43 overexpression inhibits the angiogenesis and tubulogenesis by increasing IL-6 and monocyte chemotactic protein (MCP-1) levels in MDA MB 231 (TNBC) cells [122]. In accordance with that Shao and colleagues reported gap junction dependent role of Cx43 on breast cancer and revealed that silencing of Cx43 leads to increased migration capacity in Hs578T cells and decrease vascular endothelial growth factor expression both in MDA MB 231 (TNBC) and Hs578T (TNBC) cells [12]. Cx26 enhances the extravasation while Cx43 encourages the attachment of cancer cells to the endothelial cells and diapedesis [79]. On the other hand, Cx43 hemichannels inhibits the breast cancer metastasis to the bone through ATP release. Released ATP acts as paracrine signal and triggers inflammation which leads to inhibition of the anchorage independent growth and migration of the breast cancer cells [123].

In addition to these, in accordance with clinical reports Cx26 was reported as cancer stem cell renewal factor in MDA MB 231 triple negative breast cancer cell lines through their interaction with NANOG and FAK proteins. In other point, Cx43 expression is associated with tumour growth owing to their relationship with tight and adherent junctions and their interaction with beta-catenin which is mentioned in non-canonical function of connexins. Additively, Cx26 and Cx43 associated with Bak as proapoptotic signal supported by colocalization experiments [124].

In the light of all these findings connexins cannot solely determined as pro-tumorigenic or anti-tumorigenic. All these findings suggest their context, stage and isoform dependent roles. Until to date while the role of Cx26 and Cx43 is studied both in vitro and in vivo by concerning context and stage differences, the role of Cx30 and Cx32 is poorly identified. In 2007, Koda and colleagues revealed that Cx32 expression increases the lymph node metastasis in breast cancer cells[125]. Later, Cx32 is identified in luminal epithelial cells in mammary gland and it was linked with favourable outcome in ER positive Luminal B group breast cancer patients[117] while the exact role of Cx32 in breast cancer is still elusive. However, building evidence was reported for Cx32 effect in different cancer types with its pro and anti-tumorigenic roles.

1.9 Cx32 and Cancer

Cx32 isoform which is majorly expressed in Schwann cells, oligodendrocytes and liver cells has been evaluated in several cancers while mostly studied in hepatocellular carcinoma[126]. Tumour suppressive and tumour promoter roles of Cx32 is demonstrated with its different roles and possible interactions with other proteins. In 2017 it was demonstrated that highly expressed and cytoplasmic localization of Cx32 enhances the chemoresistance and tumor growth while the same group showed anti-apoptotic effect of canonically localized Cx32 via GJIC in cervical cancer. According to these data cytoplasmic localization of Cx32 induced the expression of EGFR protein [127]. These data is important by the aspect of avoiding from the generalization of the connexins (only based on their expression level) and importance of the localization of the connexons [127]. In a study performed by Xiang and colleagues in 2019, the association between aberrant Cx32 localization in cytoplasm and advanced tumor metastasis is demonstrated in hepatocellular carcinoma. In here, it was demonstrated that Cx32 overexpression leads to chemoresistance by increasing the EGFR expression via Src kinase [128]. According to the in vivo studies performed by Liu and colleagues, Cx32's tumour favourable effect was demonstrated by indicating increased metastasis and invasion in Huh7-Cx32 xenografted mouse models [129]. Opposite to these findings it was reported that Cx32 upregulation which is mostly localized in cytoplasm reverses the epithelial to mesenchymal transition with increased E-cadherin and decreased Vimentin and Snail expression in Huh7 hepatocellular carcinoma cells [130]. In accordance with previous finding, Yang and colleagues identified that aberrant Cx32 expression in cytoplasm inhibits the invasion and metastasis through Snail mediated pathway in hepatocellular carcinoma SMMC-772 cells. Cx32 downregulation strongly associate with reduction in E-cadherin and upregulation of Snail protein with beta-catenin accumulation in nucleus. [131]. Besides, Yu and colleagues showed that Cx32 expression is strongly correlated with E-cadherin and inversely correlated with vimentin expression in doxorubicin resistant hepatocellular carcinoma HepG2 cells. In the meantime, Dox leads to downregulation of Cx32 and induction of EMTv[132]. In ovarian cancer Cx32 internalization and accumulation in cytoplasm acting a role in inhibition of apoptosis induced by cisplatin [133].

All these findings suggest that Cx32 has a critical role in cancer progression which differs according to Cx32 subcellular localization and type of the cancers. However, up

to now Cx32 status in breast cancer was evaluated with a few reports. Koda and colleagues revealed increase Cx32 expression in cytoplasm is associated with increased lymph node metastasis in breast cancer[125].

1.10 Aim of the Study

In the light of our previous studies Cx32 indicated majorly cytoplasmic and nuclear localization especially in MDA MB 231 cells. Until to now the role of cytoplasmic and nuclear localization of connexins on breast cancer are marked in several report[109, 124]. Cytoplasmic localization of Cx32 and its association with metastasis has been reported in 2006 while not any evidence was added onto that study[121, 125]. Moreover, although building evidence suggested pro-tumorigenic and anti-tumorigenic role of Cx26 and Cx43 in breast cancer[121, 134], the exact role of Cx32 on breast cancer progression still waited to be shed light on it.

In here we aimed to understand the effect of Cx32 on breast cancer. Throughout that project we evaluated and compared the localization and expression of Cx32 in mammary epithelial MCF10A and triple negative breast cancer MDA MB 231 cells. And we aimed to implicate their association with Cx32 effect on epithelial to mesenchymal transition which is a critical step in metastasis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Maintenance of MDA MB 231 and MCF10A cell

In this project triple negative breast cancer cell line, MDA MB 231 and immortalized non-tumorigenic breast cell line MCF10A cells were used. MDA MB 231 cells and MCF10A cells were grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, Cat# 41966029) and DMEM/F-12 (GIBCO, Cat# 31330038) medium, respectively. MDA MB 231 cells culture medium was supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO, Cat# 16000044) and 1% Penicillin/Streptomycin (P/S) (GIBCO, Cat # 15140122). DMEM/F-12 medium was supplemented with 5% Donor Horse Medium (DHS) (Biological Industries, Cat# 04-004-1B), 20 ng/ml EGF (Sigma, Cat# E9644), 0,5 μg/ml Hydrocortisone (Sigma, Cat# H0888), 100 ng/ml Cholera toxin (Sigma, Cat# C8052-0.5MG), 10 μg/ml Insulin (Sigma, Cat# I1882-100MG) and 1% P/S. Cells were grown in humidified atmosphere at 5% CO_2 and 37˚C. MDA MB 231 and MCF10A cells were infected with pLenti-GIII-CMV-GFP-2A-Puro referred to as pLenti-GFP or pLenti-GIII-CMV-GFP-2A-Puro-Cx32 called as pLenti Cx32 vectors.

MCF10A and MDA MB 231 cells were split in their log phase using 0,25 % Trypsin EDTA (Biological Industries, Cat# 03-053-1A) for 15 minutes and 0,05% Trypsin EDTA for 5 minutes incubation at 37˚C and 5% CO_2 humidified atmosphere, respectively.

2.2 Immunostaining and Fluorescence Analysis

MDA MB 231 and MCF10A cells were seeded on coverslips in 6 well plates and cultured for 3 days, 4 days and 7 days. While seeding the cells, it was aimed to have the same confluency of 80-90% at each selected day. In the light of this aim, optimizations were performed, and specific number of cells were seeded as shown in Table 2.1. After the incubation times of seeded cells, cells were rinsed with 1 ml 1X Phosphate Buffered

Saline (PBS) for two times and then were permeabilized with 0.1 % TritonX-100/PBS at room temperature for 15 minutes. Next, permeabilized cells were blocked in 5 % BSA in 0.1 % TritonX-100/PBS at room temperature for 45 minutes to inhibit non-specific binding. Then, cells were stained with polyclonal rabbit anti-Cx32 antibody (Invitrogen, Cat# 34-5700) in 1:200 dilution at +4°C overnight. After overnight incubation cells were rinsed with 1X PBS for three times to remove non-specific bindings and incubated with Alexa488-conjugated goat anti-rabbit secondary antibody (Sigma, Cat# ab150086) in 1:500 and DAPI (Sigma, Cat #D9542) in 1:1000 dilution for 45 minutes at room temperature and dark conditions. After the rinsing of cells with 1X PBS for three times, coverslips were dipped into dH_2O and mounted onto the slides.

For morphology analysis cells were grown on coverslips and incubated through 2 days, 3 days, 4 days and 7 days. Then, seeded cells were treated as described above and cells were incubated with phalloidin (Invitrogen, Cat# A12379) and DAPI for 45 minutes.

Then, images of the slides were taken by Olympus ix83 fluorescent microscope and analysed using Image J program.

Table 2. 1 The amount of MCF10A and MDA MB 231 cells seeded on 6 well plates.

Days	**MCF10A**	**MDA MB 231**
3 Day	$1{,}8x10^5$	$1{,}6x10^5$
4 Day	$1{,}0x10^4$	$8x10^4$
7 Day	$4x10^4$	$2x10^4$

2.3 Trypan Blue Staining/ Cell Counting

$1x10^4$ MDA MB 231 and MCF10A cells per well were cultured in 12 well plate for 7 and 9 days, respectively. On each day, cells were washed with 1X PBS and trypsinized cells were suspended in 250 µl DMEM medium. Then 50 µl cell mixture was added into 50 µl trypan blue dye followed by taking 10 µl dye-cell mixture and loading onto the haemocytometer. Counting was repeated three times per well and its average was taken. Doubling time of each cell was calculated based on their log phase according to

the formula as Doubling time (Dt) = ln2/μ where μ= ln(Total number of cells counted at last day of log phase) / ln(Total number of cells counted at initial day of log phase [135].

2.4 PI Staining for Cell Cycle Analysis

6,5x10^4 MDA MB 231 and MCF10A cell per well was seeded into 6 well plate in 2 ml. After 48 hours, 72 hours and 96 hours, cells were washed with 1X PBS. Later on, cells were trypsinized and collected in falcon tubes. Collected cells were pelleted for 10 minutes at 1,200 rpm. Consequently, supernatant was removed, and the pellet was dissolved gently with 1 ml ice cold 1X PBS on ice. Then, 4 ml ice cold 100% EtOH was added on the cell suspension and mixed gently. After that, samples were incubated at -20°C at least overnight. Next, cells were centrifuged for 10 minutes at 1,500 rpm followed by centrifugation for 1 minutes at 2,000 rpm. Then, supernatant was removed and 1 ml 1X PBS was added onto the cell pellets. After that 1X PBS cell suspensions were transferred into the Eppendorf tubes and pelleted at 1,500 rpm for 10 minutes at +4°C. Next, supernatant was aspirated and cell pellets were incubated with 200 μl 0.1 % TritonX-100/PBS and 200 μg/mL RNase for 30 minutes at 37°C after well mixing of cells with trypsin mixture. Then 20 μl of 1mg/ml PI solution was added into the samples which was incubated for 15 minutes at dark. Cell cycle analysis was performed by using FACS Canto (BD Biosciences, CA, USA).

2.5 Q-RT PCR Analysis

MDA MB 231 and MCF10A cells were seeded into the 6-well plate in 2ml medium/ well with specific counts as described in Immunostaining / Fluorescence Imaging sections. After the seeded cells were grown for 3 days, 4 days and 7 days, cells were washed with 1X PBS twice and flash frozen. Then total RNA was extracted by using PureLink® RNA Mini Kit (NucleospinRNA, Cat# 74095510) in the light of protocol provided by the manufacturer. RNA concentrations were measured by Nano-drop and cDNA synthesis was performed by using cDNA Synthesis Kit (ThermoScientific, Cat# K1622)(Vendor and catalog number) with 1μg RNA. Then cDNA samples were used for amplification with q-RT PCR with FastStart Essential DNA Green Master Mix (Roche, Cat# 42729200) which was conducted for 40 cycles on a

Roche Real Time PCR System. Afterwards cycle time (Ct) values of gene of interests were normalized to the Ct value of Tata-Box Binding Protein (TBP) and fold changes were calculated with the following formula as $2^{-\Delta\Delta Ct} = 2^{\text{control group (target gene Ct value- TBP Ct value)-experimental group (target gene Ct value- TBP Ct value)}}$. The used cycle and primer sequences are shown in Table 2.2 and Table 2.3, respectively.

Table 2. 2 Cycles used in q-RT PCR.

Stage	Temperature	Duration	Cycle
Preincubation	95°C	600 s	1 cycle
3 step amplification	95°C	30 s	45 cycle
	60°C		
	72°C		
Melting	95°C	10 s	1 cycle
	65°C	60 s	
	72°C	1 s	

Table 2. 3 Forward and Reverse Primers of genes amplified in q-RT PCR.

Gene Name	Forward Primer	Reverse Primer
Human Cx32	5'-ggcacaaggtccacatctca-3'	5'-gcatagccagggtagagcag-3'
Human TBP	5'-tagaaggccttgtgctcacc-3'	5'-tctgctctgactttagcacct-3'
E-cadherin	5'-cagcacgtacacagccctaa-3'	5'-ggtatgggggcgttgtcatt-3'
N-cadherin	5'-gacggttcgccattcaga-3'	5'-tcgattggtttgaccacg-3'
ZO-1	5'-atggaggaaacagctatatggga-3'	5'-ccaaatccaaatccaggagc-3'
Vimentin	5'-gctaaccaacgacaaagccc-3'	5'-cgttcaaggtcaagacgtgc-3'
Slug	5'-ctcctcatctttggggcgag-3'	5'-ttcaatggcatgggggtctg-3'
Snail	5'-ctaggccctggctgctacaa-3'	5'-tgtggagcagggacattcg-3'
Zeb2	5'-ataagggagggtggagtggaa-3'	5'-gttaattgcggtctggatcgtg-3'
Zeb1	5'-cccaggtgtaagcgcagaaa-3'	5'-gtctggtctgttggcaggtc-3'

2.6 Western Blot Analysis

2.6.1 Cell Lysis

Cell lysis were obtained from MCF10A and MDA MB 231 cells seeded into 6 cm plates in 5 ml medium/ plate with respective counts to 80-90% confluency as indicated in Table 2.4. After their incubation for specific days, cells medium was aspirated and rinsed with 1X PBS. After cells were trypsinized, they were centrifuged for 2 minutes at 1,200 rpm. Then supernatants were aspirated, and cell pellets were rinsed with 1 ml 1X PBS for once which is followed by addition of 100 µl lysis buffer (100 mM Tris-HCl, 1mM EDTA, 0.1% Triton X). Cell-lysis buffer mixture was mixed well and left on ice for 20 minutes. After that, cells were centrifuged at 14,000 rpm for 20 minutes at +4°C. Then supernatants were taken into Eppendorf tubes and saved for Bradford analysis.

Table 2. 4 The amount of MCF10A and MDA MB 231 cells seeded on 6 cm plates.

Days	**MCF10A**	**MDA MB 231**
3 Day	$3{,}8x10^5$	$2{,}7x10^5$
4 Day	$2x10^5$	$1{,}35x10^5$
7 Day	$5x10^4$	$3x10^4$

2.6.2 Bradford Assay

Bradford assay was composed of two distinct steps. Initially BSA (20 mg/ml) standards were prepared as 4 mg/ml, 2mg/ml, 1mg/ml, 0,5 mg/ml and 0,25 mg/ml. 800 µl autoclaved dH_2O and 200 µl Bradford reagent was mixed well into cuvettes and then 10 µl Bradford standards were added into the each 1:5 diluted Bradford reagent. The same dilutions of Bradford reagent were prepared for samples as well and as accordance with standards preparation 10 µl of protein samples were loaded into the Bradford solution. Concentration of proteins were measured at 570 nm on spectrometry.

When nucleus and cytoplasm subfractions were analysed, standards and protein samples were prepared in 1:3 diluted subcellular fractionation buffer with dH_2O to prevent the

interference of imidazole which is found in subcellular fractionation buffer with Bradford reagent.

2.6.3 Immunoblotting

SDS separating gels were prepared in 12% and 13,5% concentration for analysis of total protein and fractionation samples, respectively with 5% stacking gels. Then, protein concentrations were set as all samples concentrations have standard concentration. After that 5X loading buffer was added onto the samples followed by heating them at 95 °C for 5 minutes or 15 minutes according to the sample type. If the heated samples were total proteins incubation time was 5 minutes while if they are nucleus or cytoplasm samples they were hold for 15 minutes to prevent sticky formation in nucleus samples. Then, samples were loaded into the gel and run at 26 mA for 3 hours. After the lysates were resolved in SDS PAGE they were transferred to the PVDF membrane at 90V for 2.5 hours in ice cold temperature and blocked with 5% Milk Powder-1X Tris Buffer Saline-Tween 20 (TBS-T) solution for 1.5 hours at room temperature. Membranes were incubated with primary antibody against Cx32 (1:500; Invitrogen, Cat# 70-0600), Zeb 2 (1:1000; Sigma, Cat# SAB2108744), Snail (1:1000; CST, Cat# C15D3), Slug (1:1000; CST, Cat# C1967), E-cadherin (1:1000; CST, Cat# 24E10) ,Vimentin (1:1000; CST, Cat# D21H3) and Gamma-Tubulin (1:1000; Sigma, Cat# T65557). Incubated membranes were rinsed with 1X TBS-T solution three times for 10 minutes. Then membranes were incubated with anti-rabbit (ThermoScientific, Cat# 31460) or anti-mouse secondary antibodies (DAKO, Cat# P0447) in 1:2000 dilution. After rinsing of membranes with 1X TBS-T for 15 minutes three times, membranes were visualized by the aid of chemiluminecence (BIORAD, 1705061) with FUSION SL VILBER LOURMAT imaging system.

2.6.4 Subcellular Fractionation

Cells were seeded and collected as explained in Western Blot Analysis part and were suspended properly in 100 µl buffer (50 mM Tris pH 7.5, 150 mM NaCl,1 %Triton-X, 10 mM NaF, 10Mm Na_3VO_4). Cells-buffer mixture were then left into the incubation for 5 minutes on ice. Then cells were centrifuged at 14,000 rpm for 15 minutes. After that supernatants were saved as cytoplasm while pellets represented nucleus part. Nucleus

pellet was dissolved in 100 µl of fractionation buffer with mechanical techniques. Cytoplasmic and nuclear fractions were saved at -80°C for further analysis.

2.7 Statistical Analysis

All experiments were performed in triplicates (otherwise it was mentioned) and results were reported as mean± standard deviation. Differences between GFP and Cx32 overexpressed cells were statistically assessed by unpaired t test. The association between fold changes and different culturing days were statistically determined by one-way ANOVA- Tukey's multiple comparison test. In addition, the association between Cx32, Zeb2 expression with different culturing days were statistically assessed by two-way ANOVA – Tukey's multiple comparison test. n represents the total sample size in each experiment and p values are detailed in each figure legend with its statistical significance.

CHAPTER 3

RESULTS

3.1 SECTION I

3.1.1 Cx32 overexpression was verified in MCF10A and MDA MB 231 cells.

To address the functional significance of Cx32 on breast cancer, Cx32 overexpression was selectively ensured with Cx32-GFP constructs. Next, Cx32 overexpression was verified by Western blotting using specific antibodies against Cx32 (Figure 3.1.1). γ-tubulin was used as a loading control and Cx32 protein levels were normalized to γ-tubulin level.

Fold differences were assessed in each cell type by normalization of their protein levels to protein levels of respective control cells infected with pLenti GFP. According to the above results, Cx32 protein level increased approximately 6 times for MCF10A cells (Figure 3.1.1A; n=3, ** $p<0,01$) while Cx32 protein level increased approximately 1.5 times for MDA MB 231 cells (Figure 3.1.1B; n=3, * $p<0,05$).

3.1.2 Cx32 overexpression decreased the proliferation of MCF10A cells while did not affect the proliferation of MDA MB 231 cells.

In the literature, the role of connexins in proliferation has been reported both in gap junction dependent and independent manner. Furthermore, both proliferative and anti-proliferative role of Cx32 has been shown in different type of cells. [129, 136] In order to assess how Cx32 overexpression affect proliferation, MCF10A and MDA MB 231 cells were counted each days using trypan blue dye exclusion assay.

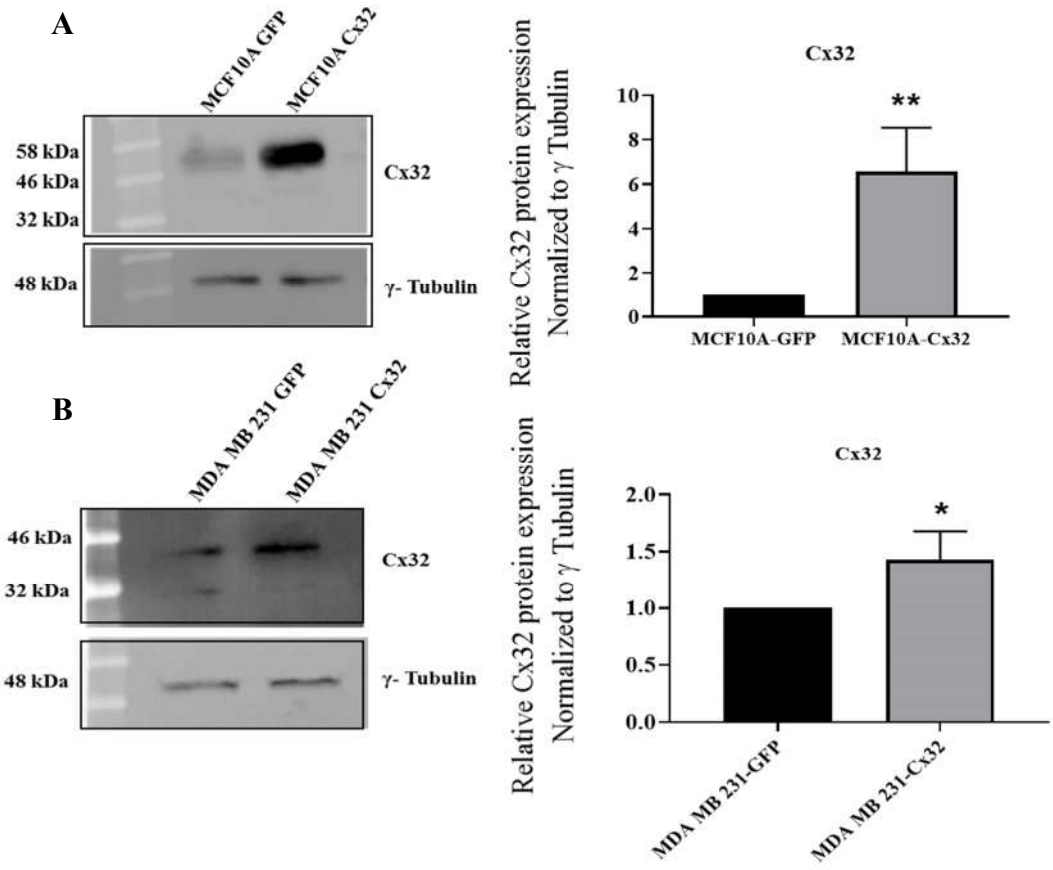


Figure 3.1.1 Verification of Cx32 overexpression both in MCF10A and MDA MB 231 cells. A. Relative Cx32 expression in MCF10A cells (n=3, ** $p<0,01$). B. Relative Cx32 expression in MDA MB 231 cells (n=3, * $p<0,05$). Statistical analysis was performed by unpaired t test. The error bars represent the standard deviation.

Doubling time of each cell type was determined by using the formula given in materials and methods considering the log phase of cells.Cx32 overexpression led to decrease in proliferation of MCF10A cells by increasing the doubling time from 21,7 hours to 27,5 hours (Figure 3.1.2A; n=3, ** $p<0,01$). However, overexpression led to slight increase in doubling time of MDA MB 231 cells which was not statistically significant (Figure 3.1.2B; n=3).

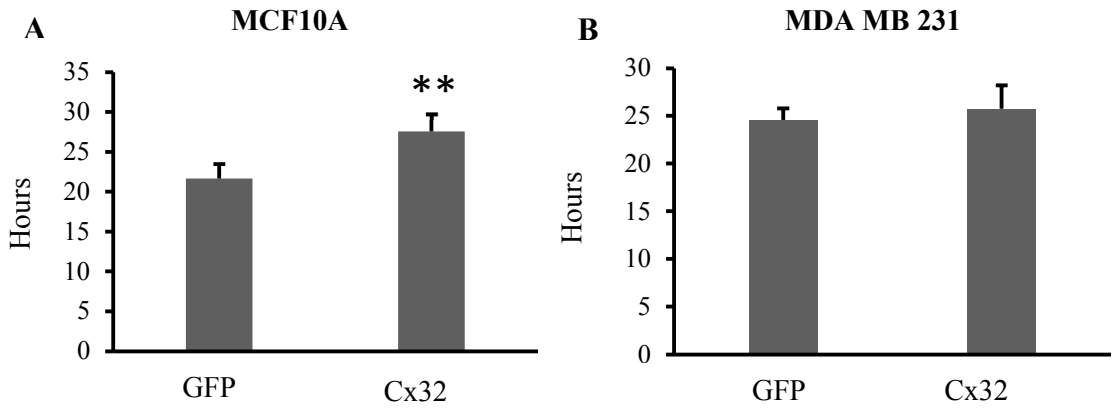


Figure 3.1.2 Doubling time of (A) MCF10A cells (n=3, ** p<0,01) (B) MDA MB 231 cells (n=3). Statistical analysis was performed by unpaired t-test. The error bars represent the standard deviation

3.1.3 Cx32 overexpression led to increase in S phase length of MCF10A cells

In order to see how Cx32 overexpression leads to increase in doubling time of MCF10A cells, PI staining was performed. PI staining was used to understand how Cx32 overexpression alter the cell cycle profile. As it was demonstrated in Figure 3.1.2, doubling time of MCF10A cells extended to 27,5 hours from 21 hours compared to control cells; that's why instead of the comparison of the percentage of cells in each cycle, times that was spent in each cell cycle was calculated and compared between groups (Figure 3.1.3 and Table 3.1.3). Moreover, MCF10A-Cx32 has a doubling time which was more than 24 hours that's why instead of focusing on just one days, we traced the cells from 2 days to 4 days.

Based on these data, length of the times that was spent in each cell cycle indicates increasing trend except G2 phase of 2 days cultured cells. According to the results, S phase of 3 days cultured cells indicated significant increase from 5,98 to 8,52 hours (Table 3.1.3; n=2, *** p<0,005) and that of 4 days cultured cells from 5,33 to 8,53 (Table 3.1.3; n=2, * p<0,05). In addition G1 phase of 3 days cultured cells indicated significant increase from 11,47 to 13,65 hours (Table 3.1.3; n=2, * p<0,05).

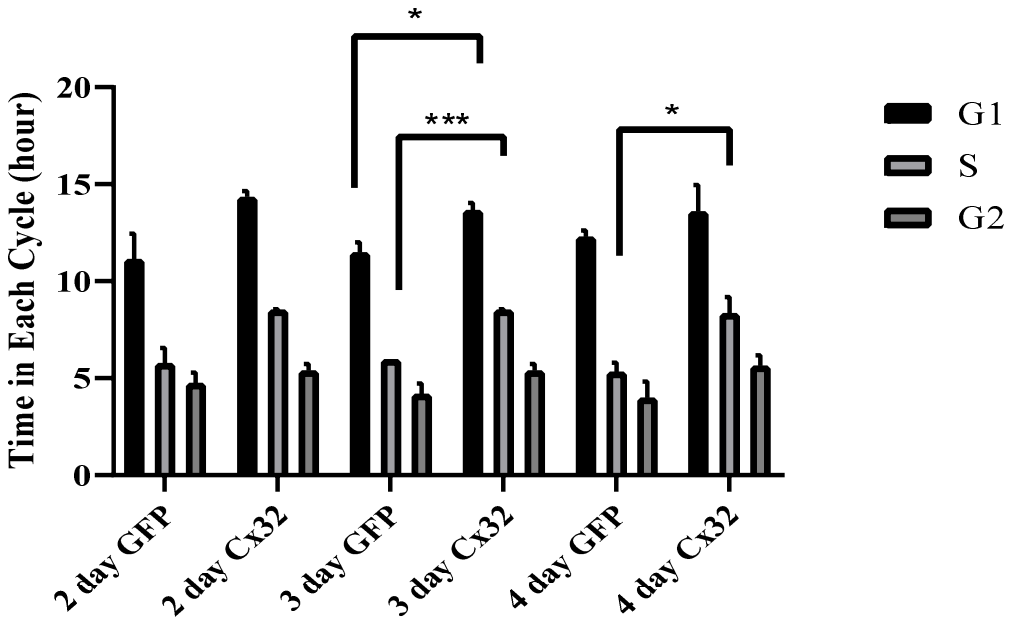


Figure 3.1.3 Time length (hours) that was spent in each cell cycle of MCF10A cells (n=2, * p<0,05 , *** p<0,005). Statistical analysis was performed by unpaired t test. The error bars represent the standard deviation.

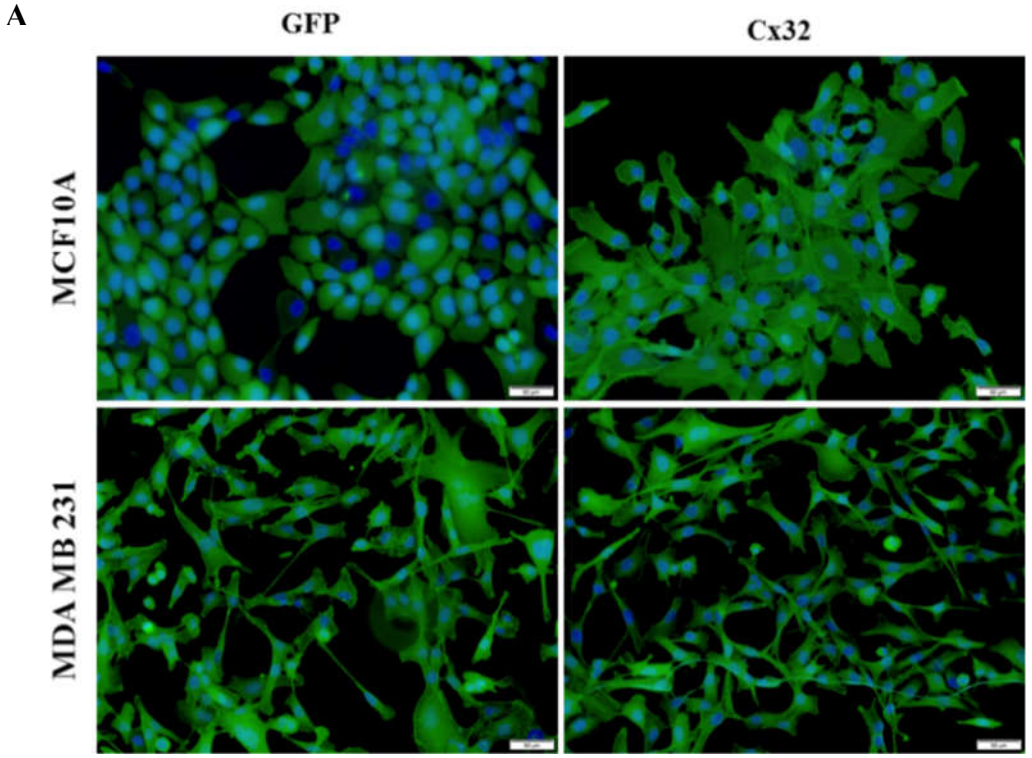
Table 3.1. 1 Time length that was spent in each cell cycle for MCF10A cells.

Culturing Time	G1 (percentage/hour)	S (percentage/hour)	G2 (percentage/hour)
2-days GFP	51,4/11,2	26,7/5,78	21,94/7,4
2-days Cx32	52/14,34	27,1/7,47	20,9/6,04
3-days GFP	53/11,47	27,6/5,98	19,4/4,2
3-days Cx32	49,5/13,65	30,9/8,52	19,5/5,38
4-days GFP	56,8/12,3	24,6/5,33	18,5/4,01
4-days Cx32	49,3/13,56	30,3/8,33	20,4/5,62

Results showed that Cx32 overexpression indicates its effect significantly in extension of G1 and S phases of cell cycle while in each days the same significance was not obtained which might be the outcome of the differences in cell cycle of the cells when they are seeded.

3.1.4 Cx32 is important for cellular morphology of MCF10A cells.

In addition to decrease in proliferation, another observable changes under the microscope was alteration of morphology with Cx32 overexpression in MCF10A cells. To assess how Cx32 affect the cell morphology, cell borders were defined by phalloidin staining with visualization of actin filaments and nuclei which were counterstained with DAPI. Morphology of each cell type was deployed by circularity index (complete circle is taken as 1) and analysis was performed in the light of measured circularity index by Image J program. Three images were analysed for each cell type in each set of experiments.



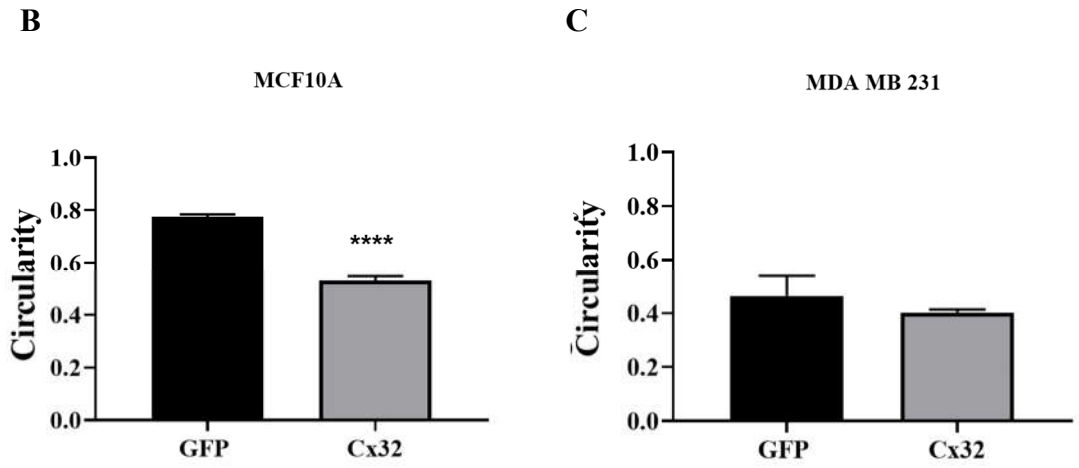


Figure 3.1. 4 Visualization and comparison of cellular morphology. (A) Visualization of MCF10A-GFP, MCF10A- Cx32 and MDA MB 231-GFP, MDA MB 231-Cx32 cells with fluorescence microscope (scale bar =50 μm). (B) Circularity index for MCF10A (n=3, **** p<0,001) and (C) MDA MB 231 cells (n=3). Statistical analysis was performed by unpaired t test.

Fluorescent images suggested that, Cx32 overexpression leads to more mesenchymal and elongated shape in MCF10A cells when compared to control cells which is also confirmed by circularity index analysis. In the light of these analysis circularity of MCF10A-Cx32 cells were reduced 27% compared to MCF10A-GFP cells (Figure 3.1.4B; n=3, **** p<0,001). However, Cx32 overexpression did not lead to any changes in cellular morphology of the MDA MB 231 cells (Figure 3.1.4C; n=3).

Until here, it was found that Cx32 is an important modulator in proliferation and morphology in cellular level in MCF10A cells while not in MDA MB 231 cells. To understand whether Cx32 lead to more mesenchymal appearance and decreased proliferation through EMT, EMT markers were investigated both in MCF10A and MDA MB 231 cells.

3.2 SECTION II

3.2.1 Cx32 indicated time and cell type dependent localization pattern.

In 2002, Polontchouk and colleagues reported days dependent expression and post-translational modification of Cx43 and Cx40 in adult rat cardiomyocytes[137]. In accordance with this finding the overexpression of Cx32 can lead to time dependent expression and localization changes which can also be varied according to the cell type. To enlighten these probabilities MCF10A-GFP, MCF10A-Cx32, MDA MB 231-GFP and MDA MB 231-Cx32 cells were stained with antibody against Cx32 and actin filaments. In addition to these nuclei were counterstained with DAPI.

Figure 3.2.1 (A) and (B) suggest that Cx32 localized in different sites in the cells such as in nucleus, cytoplasm and membrane. Moreover, localization of Cx32 indicated days and cell type dependent changes. As it was seen in Figure 3.2.1A, Cx32 is localized in nucleus in 3 days cultured MCF10A-Cx32 cells while it is disappeared from nucleus after 3-days incubation which is specifically detected in 4 days cultured MCF10A cells. On the other hand, Cx32 localized in nucleus throughout different time points in MDA MB 231 cells (Figure 3.2.1B).

Furthermore, Cx32 overexpression indicate clear increase in cytoplasmic Cx32 level in MCF10A-Cx32 cells cultured for 4 and 7 days.

Here it was suggested that majority of Cx32 did not localize in cell membrane and indicated differential localization pattern between MCF10A and MDA MB 231 cells. While Cx32 overexpression was not observed clearly from the images especially in MDA MB 231 cells, to verify the overexpression of Cx32 throughout different time points Western blotting was performed.

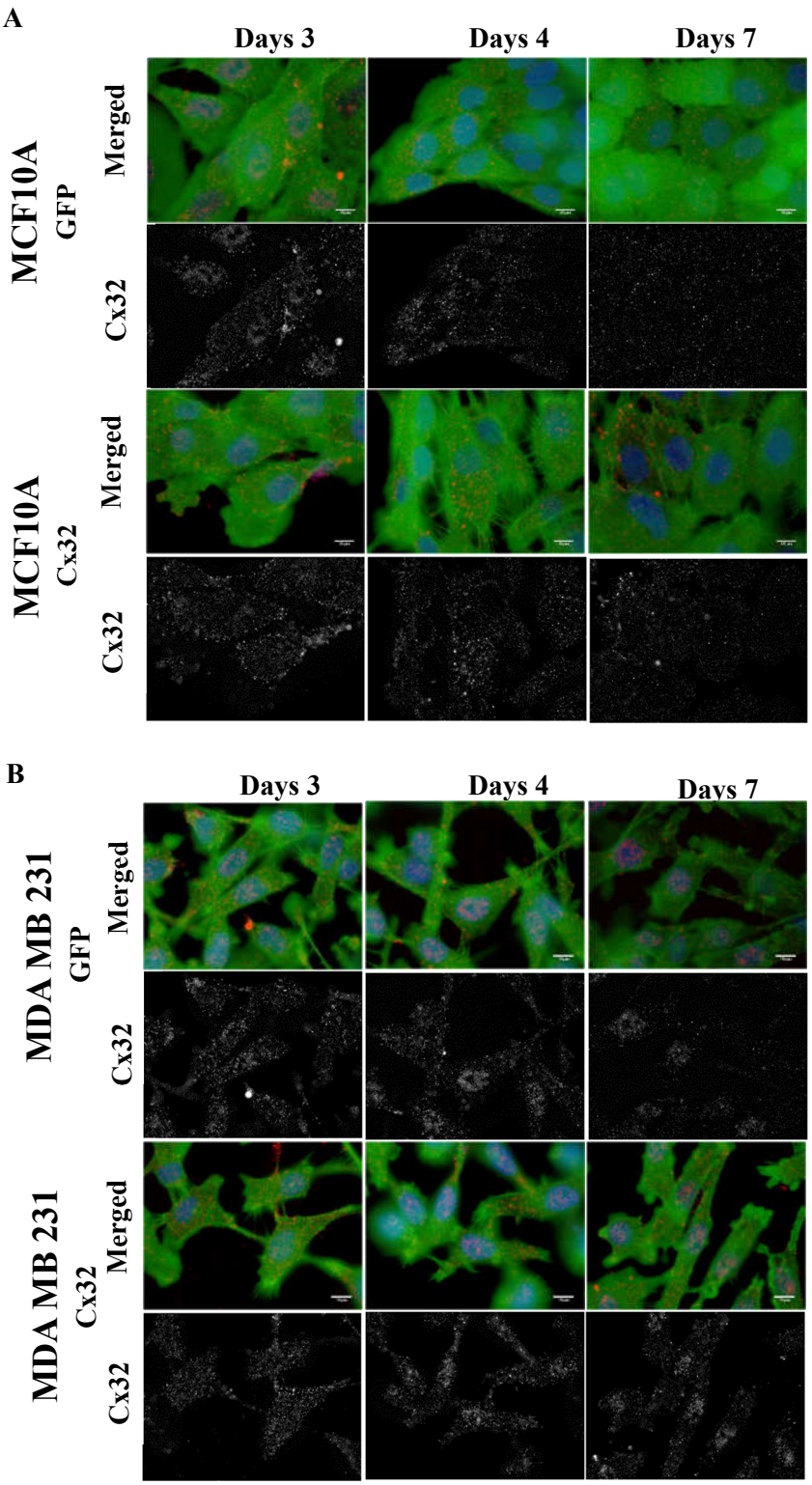


Figure 3.2.1 Localization of Cx32 in (A) MCF10A (n=2) and (B) MDA MB 231 (n=2) cells cultured in different time points (scale bar=10 μm). Cx32 is represented with red colour, actin filaments represented with green colour and nuclei were represented with blue colour.

3.2.2 Differential Cx32 expression in MCF10A and MDA MB 231 cells were verified for different time points.

In addition to differential localization and expression of Cx32, Cx32 can be found in different forms due to different post-translational modifications. To control the expression level and different form of Cx32, MCF10A and MDA MB 231 cells cultured for 3 days, 4 days and 7 days were probed with Cx32 antibodies by immunoblotting. γ-Tubulin was used as a loading control and Cx32 protein levels were normalized to them. Then, Cx32 protein levels were normalized to the GFP transfected control cells (Figure 3.2.2).

The results showed that Cx32 indicated 7.4 (Figure 3.2.2A; n=2, * $p<0,05$), 1.5 (Figure 3.2.2A; n=2, ns) and 2.5 (Figure 3.2.2A; n=2, ns) fold increase in 3 days, 4 days and 7 days cultured MCF10A-Cx32 cell, respectively with respect to control MCF10A-GFP cells. While Cx32 fold increase was statistically significant for 3 days cultured cells, it was not statistically significant for 4 days and 7 days cultured MCF10A cells (Figure 3.2.2A). In MDA MB 231-Cx32 cells Cx32 showed 3,9 fold increase for both 3 days (Figure 3.2.2B; n=2, * $p<0,05$) and 4 days (Figure 3.2.2B; n=2, * $p<0,05$) cultured cells with respect to MDA MB 231-GFP cells. However, Cx32 did not demonstrate significant changes in 7 days cultured (Figure 3.2.2B; n=2, ns) MDA MB 231-Cx32 cells with respect to control cells. Furthermore, Cx32 fold difference was significantly associated with culturing time in MCF10A cells (Figure 3.2.2A; n=2, * $p<0,05$) but not in MDA MB 231 cells (Figure 3.2.2B; n=2, ns)

Cx32 proteins fold differences were assessed for each cell with concerning the two bands aligning nearly 32 kDa and nearly 46 kDa. As seen in representative images Cx32 fold increase was mostly based on an increase in band aligned nearly 46 kDa.in MCF10A (Figure 3.2.2A). However, in MDA MB 231 cells Cx32 overexpression was mostly based on increase in form represented by band aligned around 32 kDa (Figure 3.2.2B). These differences were clearly observed in 4 days cultured MCF10A-Cx32 and MDA MB 231-Cx32 cells.

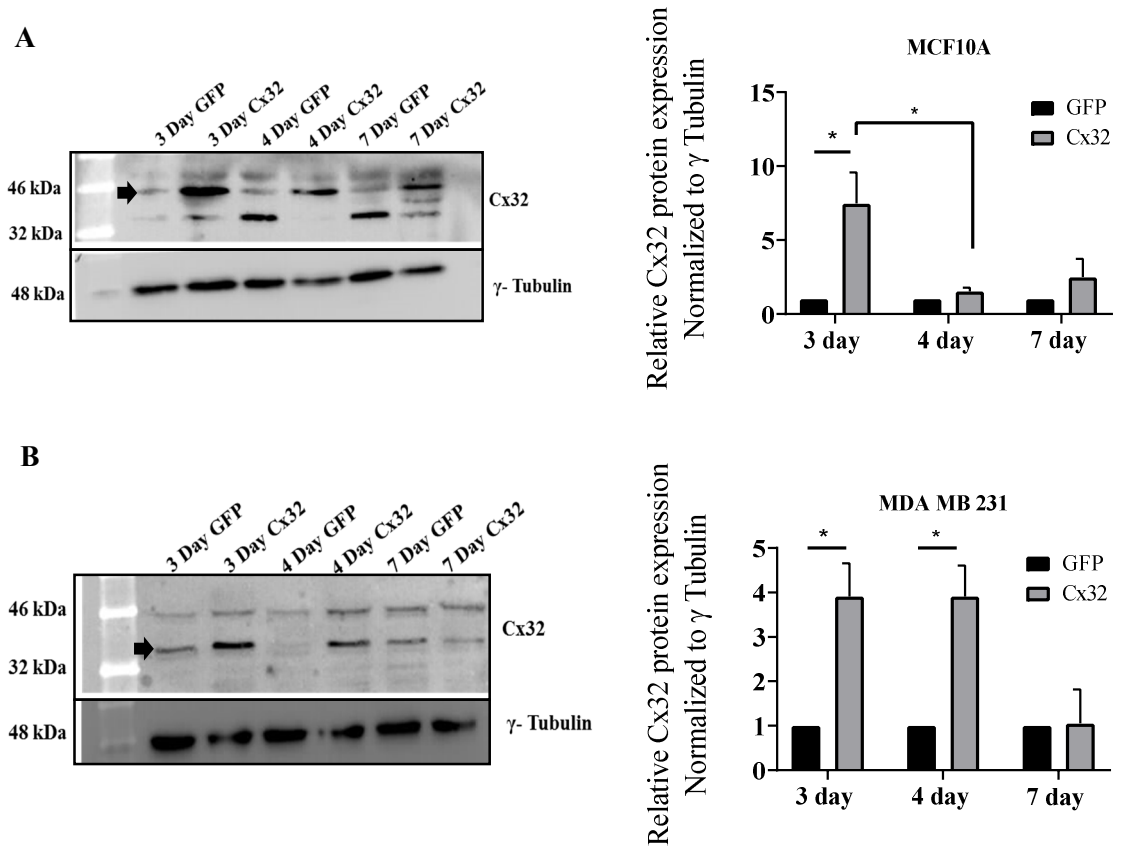


Figure 3.2.2 Verification of Cx32 protein level in MCF10A and MDA MB 231 cells cultured throughout different days. A) Cx32 fold differences with respect to control cells for 3 days (n=2, p<0,05 *), 4 days (n=2, ns) and 7 days (n=2, ns) cultured MCF10A cells and in (B) MDA MB 231 cells cultured for days (n=2, * p<0,05), 4 days (n=2, * p<0,05) and 7 days (n=2, ns). Statistical analysis was performed using unpaired t test and one-way ANOVA and Tukey's multiple comparison test (* p<0,05; ** p<0,01; *** p<0,005 ; **** p< 0,001). The error bars represent the standard deviation.

3.2.3 Nuclear localization of Cx32 was verified both for MCF10A and MDA MB 231 cells.

In Figure 3.2.1 we showed the localization of Cx32 in the cells by the aid of immunostaining and fluorescent microscopy which give us a 2D images and prevents us to do certain predictions about the localization of the Cx32.

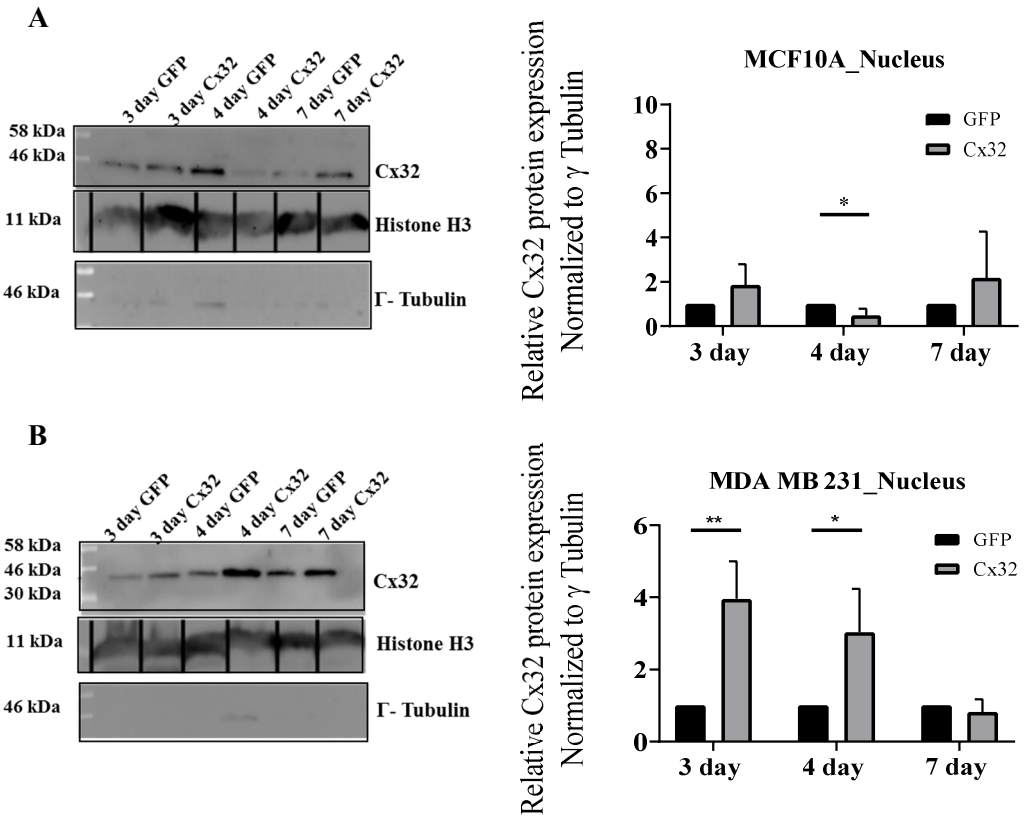


Figure 3.2.3 The nucleus fraction analysis of MCF10A and MDA MB 231 cells. (A) Cx32 localization in nucleus was verified in nucleus and increment in Cx32 protein level was verified with respect to control cells by immunoblotting for 3 days (n=3, ns), 4 days (n=3, * $p<0.05$) and 7 days (n=3, ns) cultured MCF10A cells and (B) MDA MB 231 cells cultured for 3 days (n=3, ** $p< 0.01$), 4 days (n=3, * $p<0,05$) and 7 days (n=3, ns) Statistical analysis was performed using unpaired t test and one-way ANOVA and Tukey's multiple comparison test (* $p<0,05$; ** $p<0,01$; *** $p<0,005$; **** $p< 0,001$). The error bars represent the standard deviation

To be ensure whether Cx32 localization in nucleus indicated time dependent and cell type dependent changes, subcellular fractionation method was performed to MCF10A-GFP, MCF10A-Cx32, MDA MB 231-GFP and MDA MB 231-Cx32 cells cultured for 3, 4 and 7 days.

Next, cells were probed with Cx32 antibody. Histone H3 and γ- Tubulin were used as nucleus and cytoplasm markers, respectively. Histone H3 was also used as a loading control for nucleus fractions and Cx32 protein levels were normalized to the Histone H3 level for each cell type. Each nucleus fraction was also probed with γ-Tubulin

to confirm that nucleus fraction is relieved from cytoplasm fraction mostly. For both cell type, Cx32 protein levels were normalized to the control cells which are infected with pLenti GFP construct.

According to the results (Figure 3.2.3), Cx32 was found in nucleus for both cell type and for each culturing time while not with consistent abundancy. Cx32 protein level indicated 1,8 (Figure 3.2.3A, n=3, ns) and 2,2 (Figure 3.2.3A, n=3, ns) fold increase in nucleus with respect to control cells in 3 and 7 days cultured MCF10A cells, respectively. Interestingly, it was seen that Cx32 protein level indicated nearly 50% decrease in nucleus fraction of 4 days cultured MCF10A cells (Figure 3.2.3A; n=3, * $p<0,05$). However, In Figure 3.2.1, immunostaining results suggested Cx32 protein level decreased in nucleus both in 4 days cultured MCF10A-GFP and MCF10A-Cx32 cells.

In addition to that Cx32 protein level indicated 3,9 (Figure 3.2.3B; n=3, ** $p<0,01$) and 3,0 (Figure 3.2.3B; n=3, * $p<0,05$) fold increase in nucleus with respect to control cells in 3 and 4 days cultured MDA MB 231 cells. However, Cx32 protein level did not indicate any significant changes in 7 days cultured MDA MB 231 cells (Figure 3.2.3B; n=3, ns).

Taken together, it was demonstrated that Cx32 found in nucleus for both MCF10A and MDA MB 231 cells. In addition, Cx32 overexpression led to differential Cx32 localization in nucleus between MCF10A and MDA MB 231 cells which demonstrated time dependent changes.

Furthermore, fold differences between culturing days were statistically significant between 3 days and 7 days cultured MCF10A cells (Figure 3.2.3A; n=3, * $p<0,05$).

In addition, Cx32 subcellular localization was visualized by immunofluorescence analysis by confocal microscopy. MDA MB 231-GFP and MDA MB 231-Cx32 cells stained with antibody against Cx32.

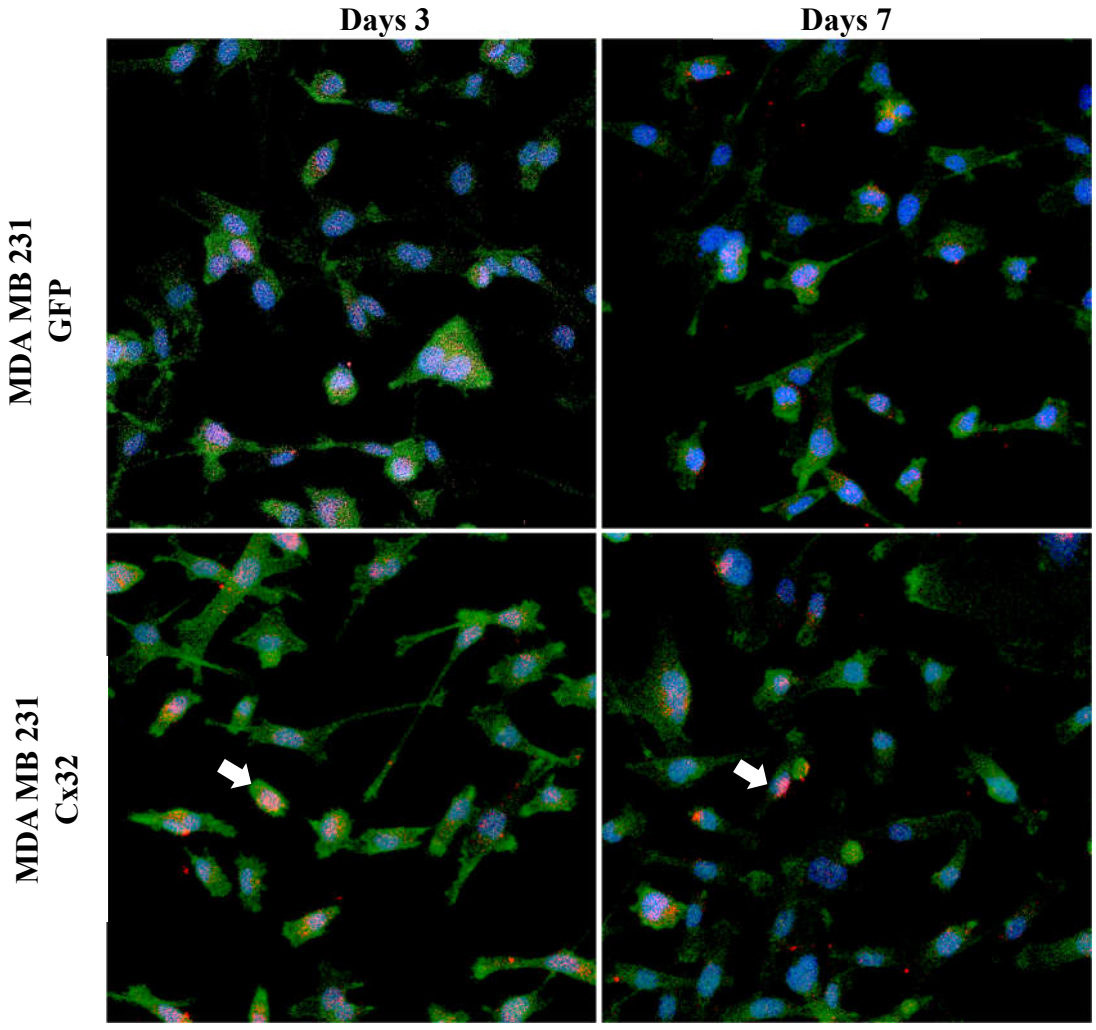


Figure 3.2.4 Confocal micrographs of MDA MB 231-GFP and MDA MB 231-Cx32 cells cultured for 3 and 7 days (40X magnification). Cx32 is represented with red colour, actin filaments represented with green colour and nuclei were represented with blue colour.

In the light of confocal micrographs (Figure 3.2.4) Cx32 overexpression was associated with nuclear localization both in 3 and 7 days cultured MDA MB 231 cells.

3.2.4 Cx32 overexpression led to decrease in E-cadherin expression at mRNA level in MDA MB 321 cells but not in MCF10A cells

In correlation with differential Cx32 expression and localization in different time points, expression of epithelial markers such as E-cadherin and ZO-1 was investigated with q-RT PCR. For both cell types the E-cadherin and ZO-1 relative expression levels were determined according to TBP housekeeping gene and then they were normalized to the E-cadherin and ZO-1 mRNA level of control cells.

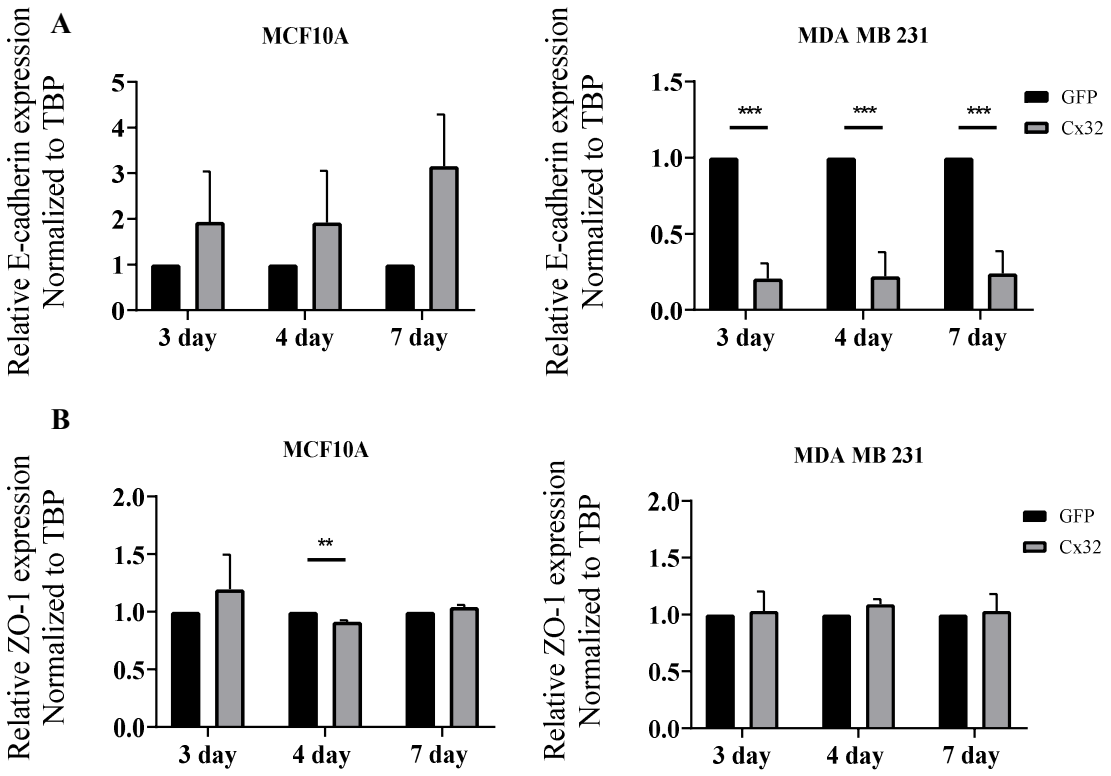


Figure 3.2.5 Relative epithelial markers expression both in MCF10A and MDA MB 231 cells. (A) Relative E-cadherin mRNA expression in MCF10A-Cx32 cells with respect to control cells cultured for 3 days (n=2, ns), 4 days (n=2, ns) and 7 days (n=2, ns) and in MDA MB 231-Cx32 cells cultured for 3 days (n=3, *** p<0,001), 4 days (n=3, *** P<0,001) and 7 days (n=3, *** P<0,001) (B) Relative ZO-1 mRNA expression for 3 days (n=2, ns), 4 days (n=2, * p<0,05) and 7 days (n=2, ns) cultured MCF10A cells compared to control cells. Relative ZO-1 expression did not indicate any significant changes (n=3) in MDA MB 231 cells. Statistical significance was assessed by unpaired t test and one way- ANOVA and Tukey's multiple comparison test (* p<0,05; ** p<0,01; *** p<0,005; **** p< 0,001). The error bars represent the standard deviation.

According to the results, mRNA levels of E-cadherin increased 1,9 fold both in 3 day (Figure 3.2.5A; n=3, ns) and 4 day (Figure 3.2.5A; n=2, ns) cultured MCF10A-Cx32 cells with respect to MCF10A-GFP control cells. In addition, E-cadherin expression increased 3,2 fold (Figure 3.2.5A; n=2, ns) in 7 day cultured MCF10A cells. Unlike to MCF10A cells, E-cadherin mRNA level expression indicated 80% (Figure 3.2.5A; n=3, *** p<0,005), 78% (Figure 3.2.5A; n=3, *** p<0,005) and 76% (Figure 3.2.5A; n=3, *** p<0,005) decrease for 3, 4 and 7 day cultured MDA MB 231 cells, respectively. Furthermore, not any significant relation was estimated between the day and fold differences both in MCF10A and MDA MB 231 cells.

Moreover, the mRNA level of ZO-1 significantly decreased by 10% (Figure 3.2.5B; n=2, ns) in 4 day cultured MCF10A-Cx32 cells compared to MCF10A GFP cells. In addition, no significant changes were observed in neither MCF10A cells cultured for 3 (Figure 3.2.5B; n=2, ns) nor 7 days (Figure 3.2.5B; n=2, ns) and in MDA MB 231 cells cultured for 3 (Figure 3.2.5B; n=3, ns), 4 (Figure 3.2.5B; n=3, ns) and 7 days (Figure 3.2.5B;n=3, ns). In accordance with E-cadherin expression level, not any significant relation was estimated between the day and fold differences both in MCF10A and MDA MB 231 cells in ZO-1 expression.

3.2.5 Cx32 overexpression led to opposing affect in E-cadherin protein level between MCF10A and MDA MB 231 cells.

Q-RT PCR results showed that Cx32 overexpression leads to significant decrease in E-cadherin expression in MDA MB 231 cells (Figure 3.2.5A). However in MCF10A cells, E-cadherin level increased independent from the culturing time even though it was not significant (Figure 3.2.5A) To confirm changes in E-cadherin expression, E-cadherin protein level was controlled by Western blotting for both MCF10A and MDA MB 231 cells cultured for 3 day, 4 day and 7 days. γ-Tubulin was used as loading control and fold differences was assessed by normalization of Cx32 overexpressing cells to control cells infected by pLenti-GFP constructs.

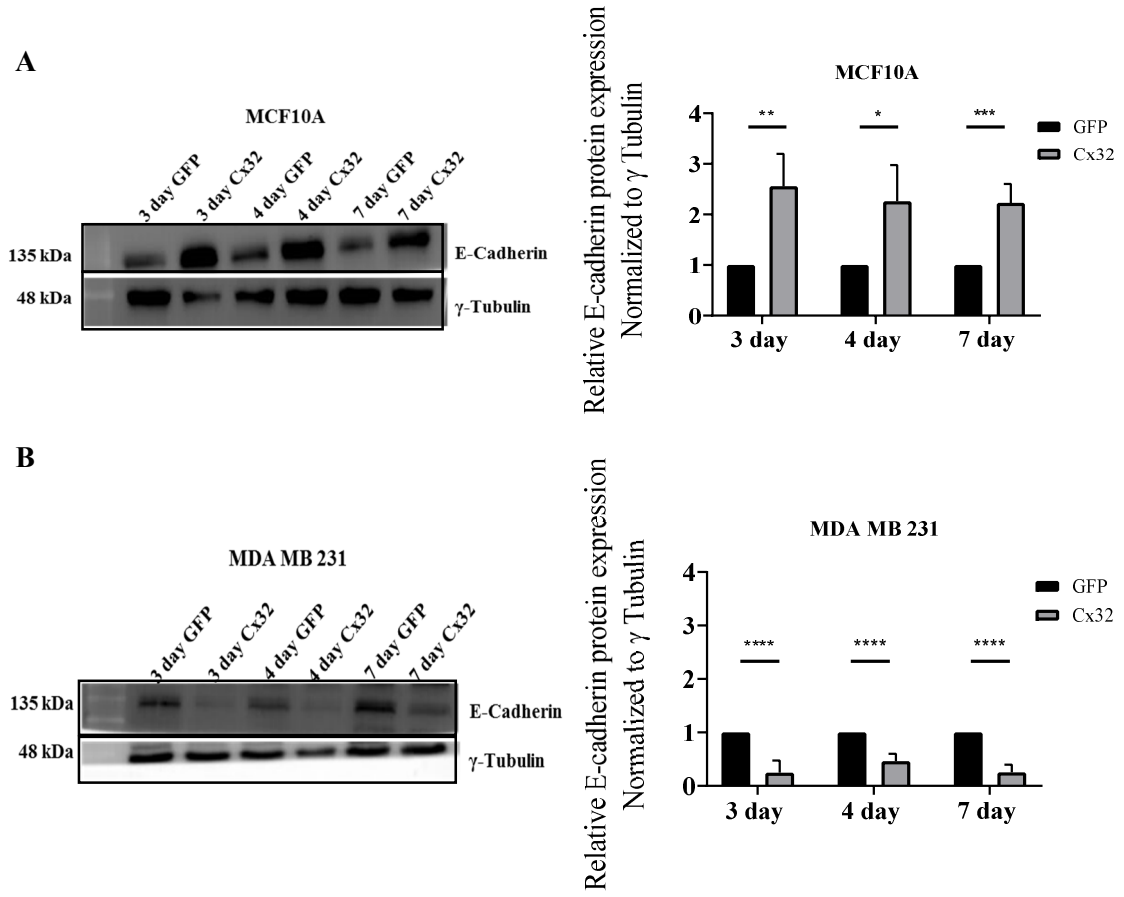


Figure 3.2.6 E-cadherin protein expression level for MCF10A and MDA MB 231 cells. (A) Relative E-cadherin protein expression level for 3 day (n=3,** p<0,01), 4 day (n=3, * p<0,05) and 7 day (n=3, *** p<0,005) cultured MCF10A-Cx32 cells with respect to control cells. (B) Relative E-cadherin protein expression level for 3 day (n=3, **** p<0,001), 4 day (n=3, **** p<0,001) and 7 day (n=3, **** p<0,001) cultured MDA MB 231-Cx32 cells with respect to control cells. Statistical significance was assessed by un-paired t test and one way ANOVA and multiple comparison Tukey test (* p<0,05; ** p<0,01; *** p<0,005; **** p< 0,001). The error bars represent the standard deviation.

E-cadherin expression significantly increased 2,5 (Figure 3.2.6A; n=3, **p<0,01), 2,3 (Figure 3.2.6; n=3, * p<0,05) and 2,2 (Figure 3.2.6A; n=3, *** p<0,005) fold for 3 day, 4 day and 7 day cultured MCF10A-Cx32 cells with respect to control cells, respectively. In contrast, Cx32 overexpression led to 90% (Figure 3.2.6B; n=3, ***** p<0,001) , 59% (Figure 3.2.6B; n=3, **** p <0,001) and 90% (Figure 3.2.6B n=3, **** p<0,001) decrease for 3 day, 4 day and 7 day cultured cells, respectively which are in agreement with q-RT PCR results. Furthermore, in accordance with q-RT PCR results,

E-cadherin expression did not indicate significant changes in protein level depending on culturing time either in MCF10A or MDA MB 231 cells.

Overall, Western blotting and q-RT PCR results suggest opposite effect of Cx32 overexpression on E-cadherin expression between MDA MB 231 cells and MCF10A cells.

3.2.6 E-cadherin localization pattern was visualized in MCF10A cells

Based on the elevated level of E-cadherin in MCF10A, to understand whether localization pattern of E-cadherin was also influenced, cells were stained with antibody against E-cadherin. Nuclei were counterstained with DAPI.

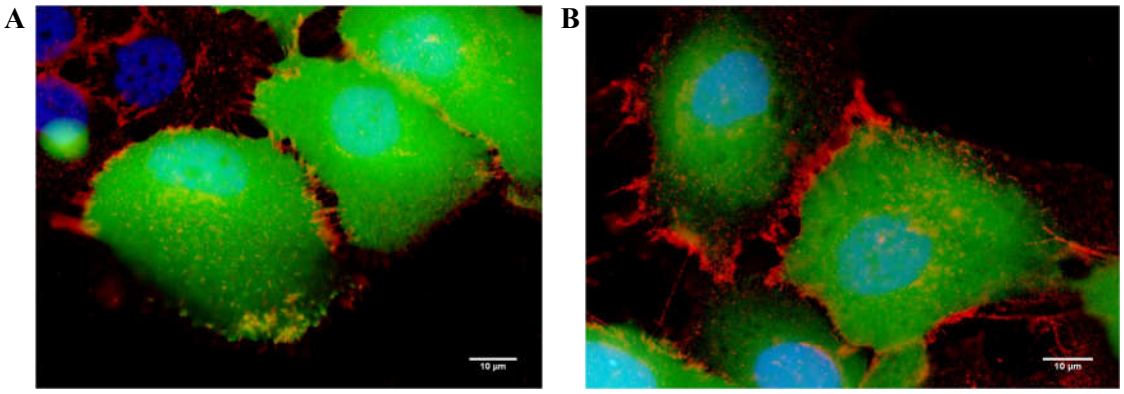


Figure 3.2.7 Localization of E-cadherin in a) MCF10A-GFP and b) MCF10A-Cx32 cells. Images were taken at 100X magnification (scale bar=10 µm, n=2)

In Figure 3.2.7A and in Figure 3.2.6B E-cadherin localization was observed in cell to cell contact sites and in accordance with Western blot results (Figure 3.2.6A), in Figure 3.2.7B elevated E-cadherin level was observed in Cx32 overexpressing cells.

3.2.7 Cx32 overexpression led to significant increase in N-cadherin expression at mRNA level in MDA MB 231 cells while Vimentin expression indicated significant increase only in 7 day cultured MDA MB 231 cells.

Next, we asked how Cx32 overexpression affect the expression of mesenchymal markers, expression of N-cadherin and vimentin were investigated with q-RT PCR. For both cell types the N-cadherin and Vimentin relative expression levels were estimated according to TBP housekeeping gene and then they were normalized to the N-cadherin and Vimentin mRNA level of control cells.

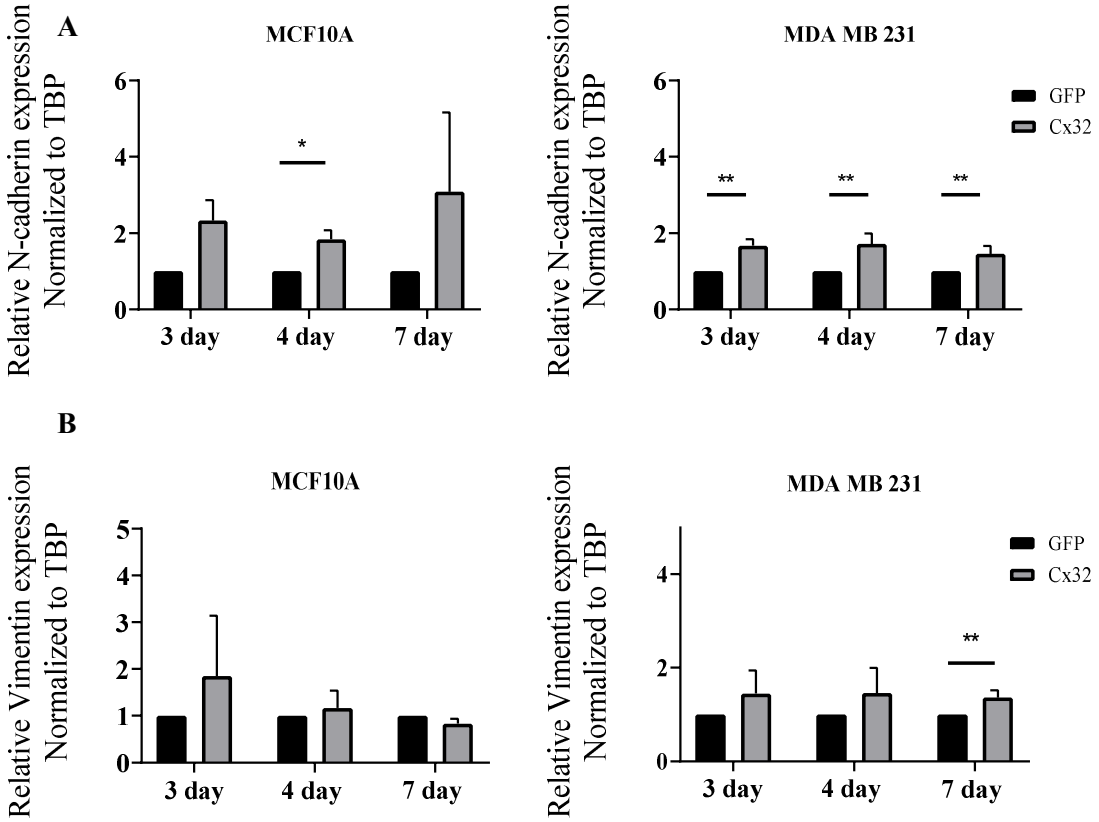


Figure 3.2.8 Relative mesenchymal markers expression both in MCF10A and MDA MB 231 cells. (A) Relative N-cadherin mRNA expression in MCF10A-Cx32 cells with respect to control cells cultured for 3 day (n=2, ns), 4 day (n=2, * p<0,05) and 7 day (n=2, ns) and in MDA MB 231-Cx32 cultured for 3 day (n=3, ** p<0,01), 4 day (n=3, ** p<0,01) and 7 day (n=3, ** p<0,01).

Figure 3.2 8 (cont.) (B) Relative Vimentin mRNA expression for 3 day, 4 day and 7 day cultured MCF10A cells did not indicate any significant changes and in MDA MB 231 cells cultured for 3 day (n=3, ns), 4 day (n=3, ns) and 7 day (n=3, ** p<0,01). Statistical significance was assessed by unpaired t test and one way ANOVA Tukey's multiple comparison test (* p<0,05 ; ** p<0,01; *** p<0,005; **** p< 0,001). The error bars represent the standard deviation.

According to the results, mRNA level of N-cadherin was increased 2.3 (Figure 3.2,8A; n=2, ns); 1,8 (Figure 3.2,8A; n=2, * p<0,05) and 3.1 (Figure 3.2,8A; n=2, ns) fold in 3 day, 4 day and 7 day cultured MCF10A-Cx32 cells, respectively with comparison to control cells while only 4 day cultured cells indicated significant increment. For MDA MB 231 cells mRNA level of N-cadherin indicated significant increases as 1,6 (Figure 3.2.8A; n=3, ** p<0,01), 1,7 (Figure 3.2,8A; n=3, ** p<0,01) and 1,4 (Figure 3.2,8A; n=3, ** p<0,01) fold respectively for 3 day, 4 day and 7 day cultured cells. Furthermore, Vimentin expression of MCF10A-Cx32 indicated 1,8 (Figure 3.2,8B; n=2, ns) fold increase in 3 day cultured cells while did not indicate any significant changes in 4 and 7 day cultured cells with respect to control cells, while in MDA MB 231 cells Cx32 overexpression did not lead to significant changes in Vimentin mRNA level.

Both MCF10A (n=2, ns) and MDA MB 231 (n=3, ns) cells did not indicate significant relationship between culturing time and N-cadherin and Vimentin expressions.

3.2.8 Vimentin expression increased in protein level for MCF10A cells but not in MDA MB 231 cells

In several reports, strong correlation between Cx32 and Vimentin has been indicated [128, 132-133]. In spite of no significant changes were observed at mRNA level (except 7 day cultured MDA MB 231 cells), in here we confirmed the protein level of Vimentin in 3 day, 4 day and 7 day cultured MCF10A and MDA MB 231 cells (Figure 3.2.9)

According to the results it was shown that Vimentin protein level increased 1,7 fold (Figure 3.2.9A; n=3, ns), 3,8 fold (Figure 3.2.9A; n=3, ** p<0,01) and 2,8 fold (Figure 3.2.9A; n=3, ** p<0,01) for 3 day, 4 day and 7 day cultured MCF10A-Cx32 cells respectively.

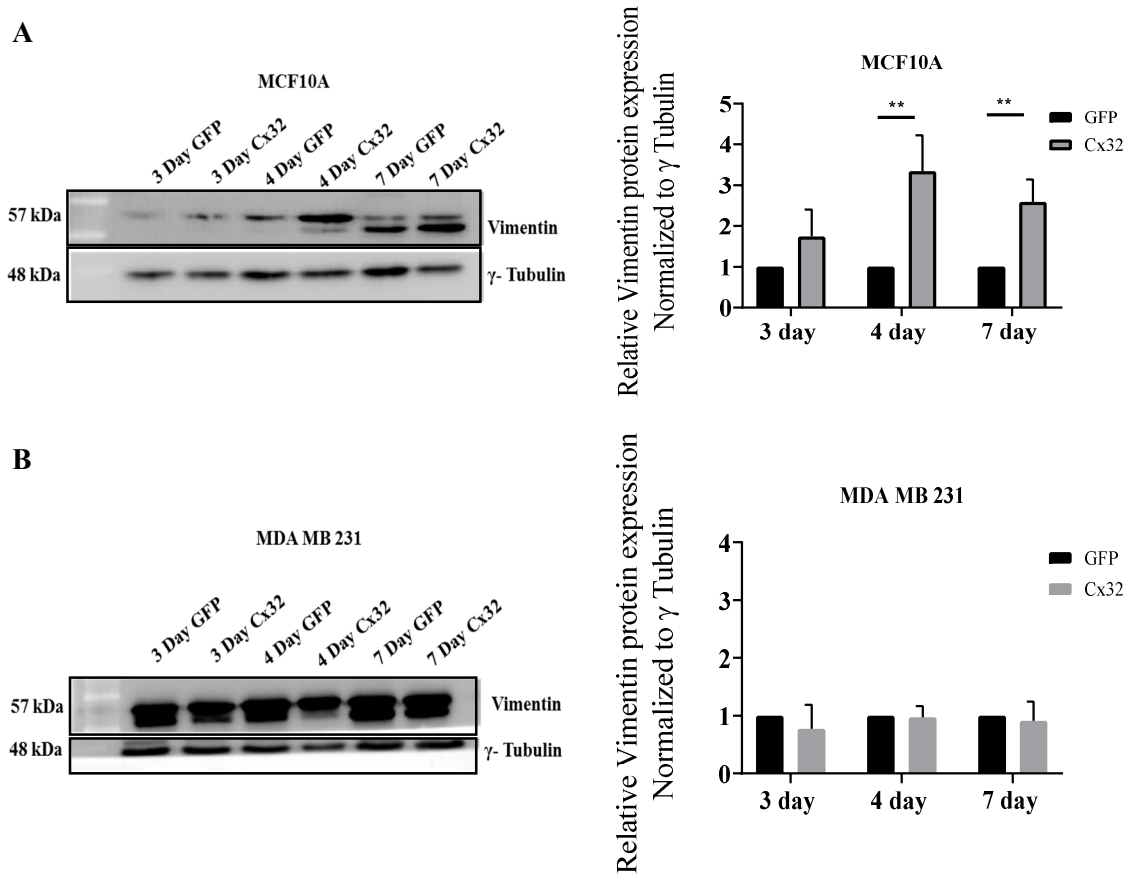


Figure 3.2.9 Vimentin protein expression level for MCF10A and MDA MB 231 cells. (A) Relative Vimentin protein expression level for 3 day (n=3, ns) , 4 day (n=3, ** p<0,01) and 7 day (n=3, ** p<0,01) cultured MCF10A-Cx32 cells with respect to control cells. (B) Relative Vimentin protein expression level for 3 day (n=3, ns), 4 day (n=3, ns) and 7 day (n=3, ns) cultured MDA MB 231-Cx32 cells with respect to control cells. Statistical significance was assessed by un-paired t test and one-way ANOVA and multiple comparison Tukey test (* p<0,05; ** p<0,01; *** p<0,005; **** p< 0,001). The error bars represent the standard deviation.

Unlike to q-RT PCR results, significant increases were observed in 4 day and 7 day cultured MCF10A-Cx32 cells. On the other hand, no significant changes were observed in Vimentin protein level of MDA MB 231 cells (Figure 3.2.9B; n=3, ns). Moreover, in accordance with q-RT PCR results Vimentin protein level did not indicate relationship with culturing times both in MCF10A (n=3, ns) and MDA MB 231 cells (n=3, ns).

Until here, our study suggested Cx32 overexpression leads to decrease in epithelial marker E-cadherin in MDA MB 231 cells while in MCF10A cells Cx32

overexpression leads to increase in E-cadherin expression with an increase in mesenchymal marker, Vimentin expression.

3.2.9 SNAI Family transcription factors expression was affected at mRNA level with Cx32 overexpression

Next, to determine which driving forces taking roles behind the changes in epithelial or mesenchymal markers, expression levels of SNAI family transcription factors were investigated with q-RT PCR. For both cell types the Snail and Slug relative expression levels were estimated according to TBP housekeeping gene and then they were normalized to the Snail and Slug mRNA level of control cells.

Q-RT PCR results showed that Cx32 overexpression did not lead to significant changes in MCF10A cells. For MDA MB 231 cells, Cx32 overexpression led to significant decrease in 3 day, 4 day and 7 day cultured cells. Snail expression was decreased 62% (Figure 3.2.10A; n=3, ** $p<0,01$), 58% (Figure 3.2.10A; n=3, *** $p<0,005$) and 52% (Figure 3.2.10A; n=3, **** $p<0,001$) in 3 day, 4 day and 7 day cultured MDA MB 231 cells, respectively.

As another SNAI family member, Slug expression indicated 1,3 fold (Figure 3.2.10B; n=2, * $p<0,05$) significant increase in 3 day cultured MCF10A-Cx32 cells compared to control cells. However, no significant changes were observed in 4 day and 7 day cultured MCF10A cells. Furthermore, results showed that Slug expression indicated significant relationship with culturing time between 3 and 7 days (Figure 3.2.10B n=2, * $p<0,05$). On the other hand in MDA MB 231 cells Slug expression did not indicate relation with time and indicated 1,8 (Figure 3.2.10B; n=3, * $p<0,05$) , 2,1 (Figure 3.2.10B; n=3, * $p<0,05$) and 1,5 (Figure 3.2.10B n=3, ns) fold increase in 3 day, 4 day and 7 day cultured cells respectively with respect to control cells.

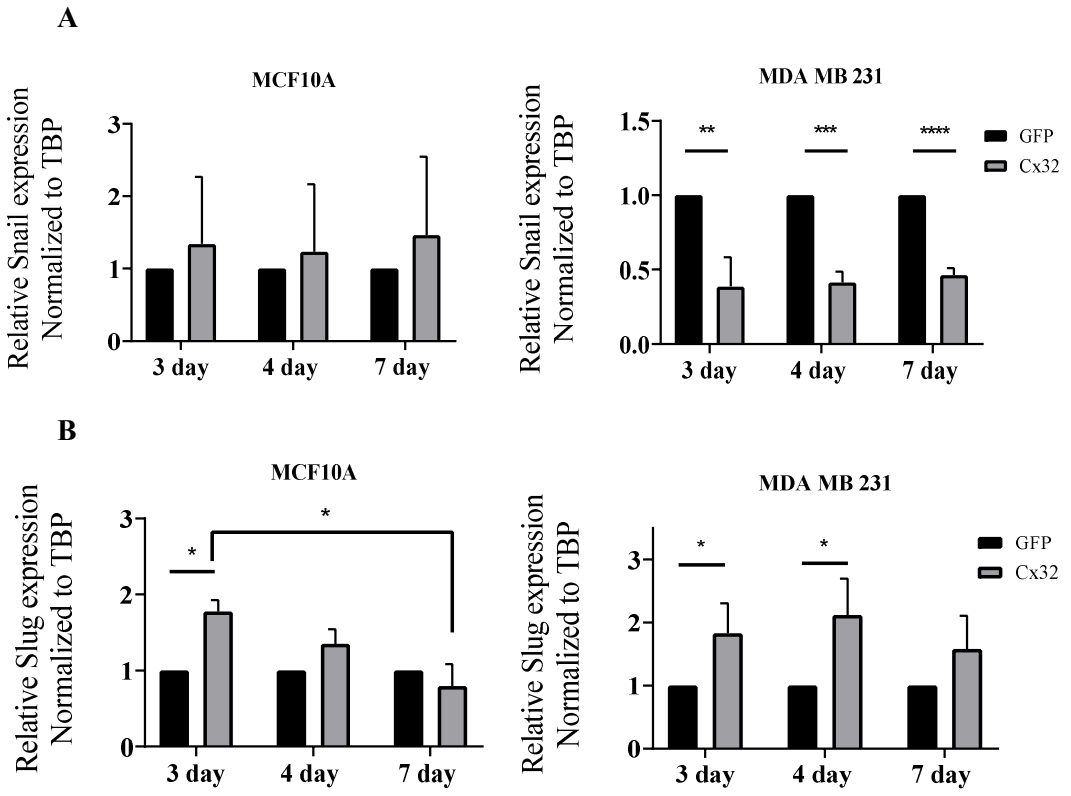


Figure 3.2.10 Relative SNAI family transcription factors expression level both in MCF10A and MDA MB 231 cells. (A) Relative Snail mRNA expression in MCF10A-Cx32 cells with respect to control cells cultured for 3 day (n=2, ns), 4 day (n=2, ns) and 7 day (n=2, ns) and in MDA MB 231-Cx32 cells cultured for 3 day (n=3, ** p<0,01), 4 day (n=3, *** P<0,005) and 7 day (n=3, **** P<0,001) (B) Relative Slug mRNA expression in MCF10A-Cx32 cells with respect to control cells for 3 day (n=2, * p<0,05), 4 day (n=2, ns) and 7 day (n=2, ns) and in MDA MB 231 cells for 3day (n=3, * p<0.05), 4 day (n=3, * p<0.05) and 7 day cultured cells.Statistical significance was assessed by unpaired t test and one way ANOVA and multiple comparison Tukey test (* p<0,05; ** p<0,01; *** p<0,005; **** p< 0,001). The error bars represent the standard deviation.

3.2.10 Cx32 overexpression significantly reduced the Snail protein in MDA MB 231 cells

Depending on q-RT PCR results (Figure 3.2.10), Cx32 overexpression led to significant decrease in Snail expression at mRNA level in MDA MB 321- Cx32 cells with respect to control cells. However, no significant change was observed in MCF10A cells. In accordance with RNA analysis, MDA MB 231-Cx32 cells indicated 70% (Figure 3.2.11B; n=3, **** p<0,001), 85% (Figure 3.2.11B; n=3, **** p<0,001) and 65% (Figure 3.2.11B; n=3, **** p<0,001) decrease in 3 day, 4 day and 7 day cultured cells

respectively. However, MCF10A cells did not indicate any significant changes in Snail level for 3 and 4 day cultured cell while as seen in MDA MB 231 cells, in MCF10A-Cx32 cells, 3,5 fold (Figure 3.2.11A; n=2, **** p<0,001) decrease was shown in 7 day cultured cells compared to control cells. In accordance with q-RT PCR results Snail protein level did not indicate significant relationship with different culturing times both in MCF10A (Figure 3.2.11A; n=2, ns) and in MDA MB 231 cells (Figure 3.2.11B; n=3, ns)

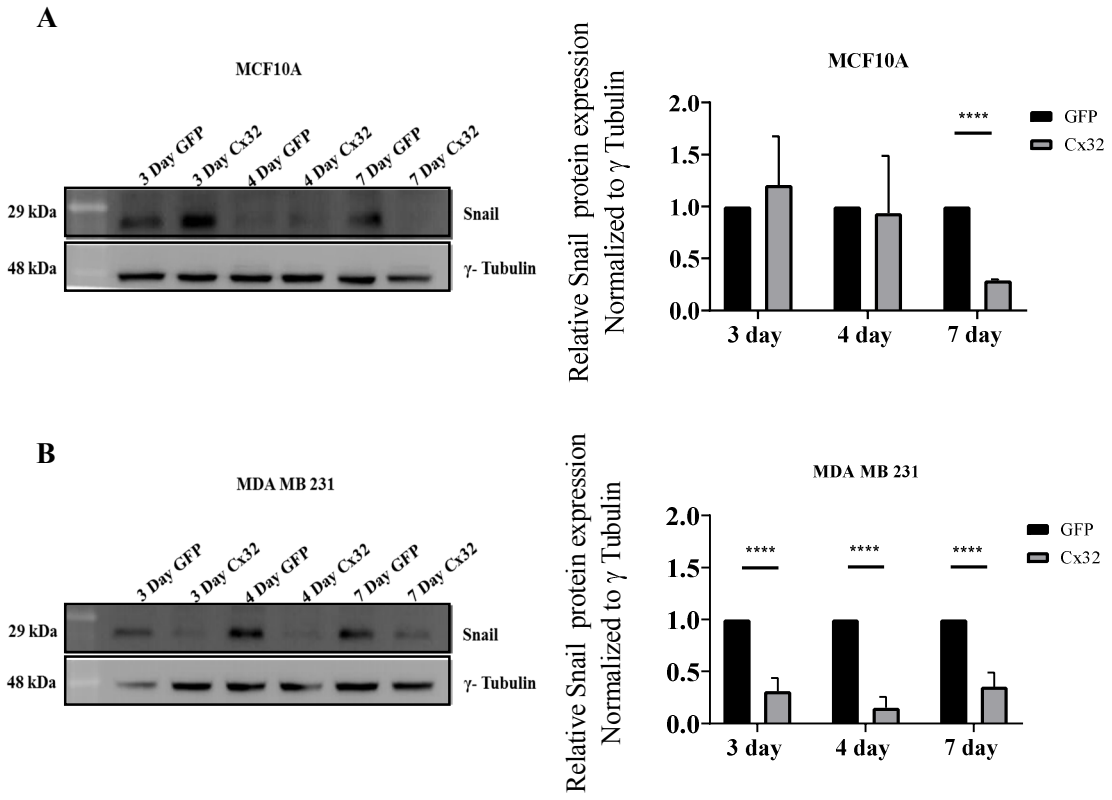


Figure 3.2. 11 Snail protein expression level for MCF10A and MDA MB 231 cells. (A) Relative Snail protein expression level for 3 day (n=2, ns) , 4 day (n=2, ns) and 7 day (n=2, **** p<0,001) cultured MCF10A-Cx32 cells with respect to control cells (B) Relative Snail protein expression level for 3 day (n=3, **** p<0,001), 4 day (n=3, **** p<0,001) and 7 day (n=3, **** p<0,001) cultured MDA MB 231-Cx32 cells with respect to control cells. Statistical significance was assessed by unpaired t test and one-way ANOVA and multiple comparison Tukey test (* p<0,05; ** p<0,01; *** p<0,005; **** p< 0,001). The error bars represent the standard deviation.

Overall Snail expression is significantly affected from the Cx32 overexpression both in mRNA and protein level in MDA MB 231 cells but not in MCF10A cells. In

MCF10A cells significant changes was only observed in 7 day cultured cells which may suggest time dependent effect of Cx32 while it was not statistically significant.

3.2.11 Cx32 overexpression led to significant increase in Slug protein only in 4 day cultured MDA MB 231 cells.

In the light of q-RT PCR results Slug indicated day dependent expression in MCF10A-Cx32 cells compared to control cells (Figure 3.2.10B). To confirm changes in Slug protein level through different culturing time and with Cx32 overexpression, protein level of Slug was investigated both in MCF10A and MDA MB 231 cells.

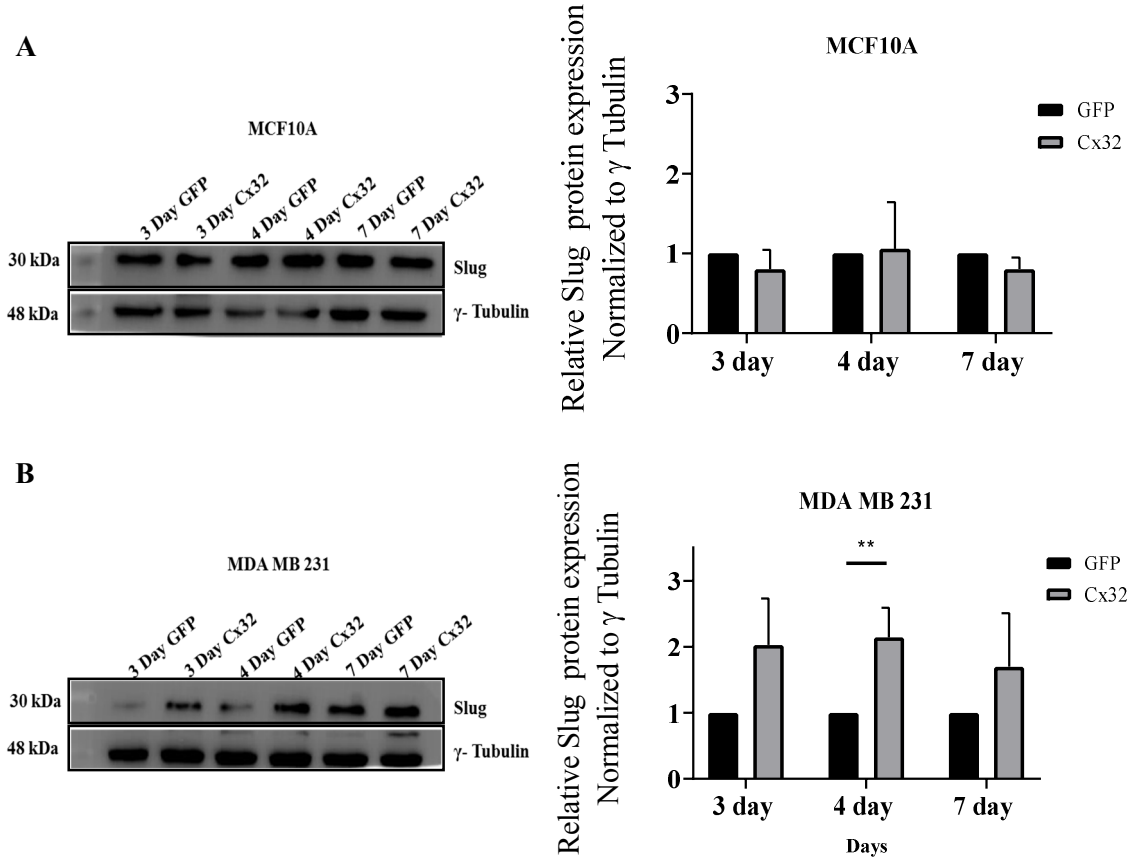


Figure 3.2. 12 Slug protein expression level for MCF10A and MDA MB 231 cells. (A) Relative Slug protein expression level for 3 day (n=3, ns), 4 day (n=3, ns) and 7 day (n=3, ns) cultured MCF10A-Cx32 cells with respect to control cells

Figure 3.2.12 (cont.) (B) Relative Slug protein expression level for 3 day (n=3, ns), 4 day (n=3,** p<0,01) and 7 day (n=3, ns) cultured MDA MB 231-Cx32 cells with respect to control cells. Statistical significance was assessed by unpaired t test and one-way ANOVA and multiple comparison Tukey test (* p<0,05; ** p<0,01; *** p<0,005; **** p< 0,001). The error bars represent the standard deviation.

According to the results Cx32 overexpression did not lead to significant changes in Slug protein level in MCF10A-Cx32 cells. Moreover, Cx32 overexpression indicated 2 fold (Figure 3.2.12B; n=3, ns), 2,1 fold (Figure 3.2.12B; n=3, ** p<0,01) and 1,7 fold (Figure 3.2.12B; n=3, ns) increase in Slug for 3 day,4 day and 7 day cultured in MDA MB 231-Cx32 cells with respect to control cells, respectively.

Overall Cx32 did not affect Slug expression in MCF10A cells while in MDA MB 231 cells Slug had an increased trend in different time points. Moreover, Slug expression did not indicate day dependent changes in MDA MB 231 (Figure 3.2.12B; n=3, ns) and in MCF10A (Figure 3.2.12A; n=3, ns) cells.

3.2.12 ZEB family transcription factors expression was affected with Cx32 overexpression

After verification of SNAI family transcription factor expressions, ZEB family transcription factors expressions were investigated by q-RT PCR. For both cell types Zeb2 and Zeb1 relative expression levels were estimated according to TBP housekeeping gene and then they were normalized to the Zeb2 and Zeb1 mRNA level of control cells.

In the light of results relative Zeb2 mRNA expression increased 2,4 (Figure 3.2.13A; n=2, p<0,05*) and 1,7 fold (Figure 3.2.13A n=2, ns) for 3 day and 4 day cultured MCF10A-Cx32 cells with respect to control cells, respectively. However, 34% (Figure 3.2.13A; n=2, ** p<0,01) decrease was observed in 7 day cultured MCF10A-Cx32 cells with comparison to control cells. Cx32 overexpression led to 1,3 fold increase in Zeb2 expression both in 4 (Figure 3.2.13A; n=3, * p<0,05) and 7 day (Figure 3.2.11A; n=3, * p<0,05) cultured MDA MB 231 cells.

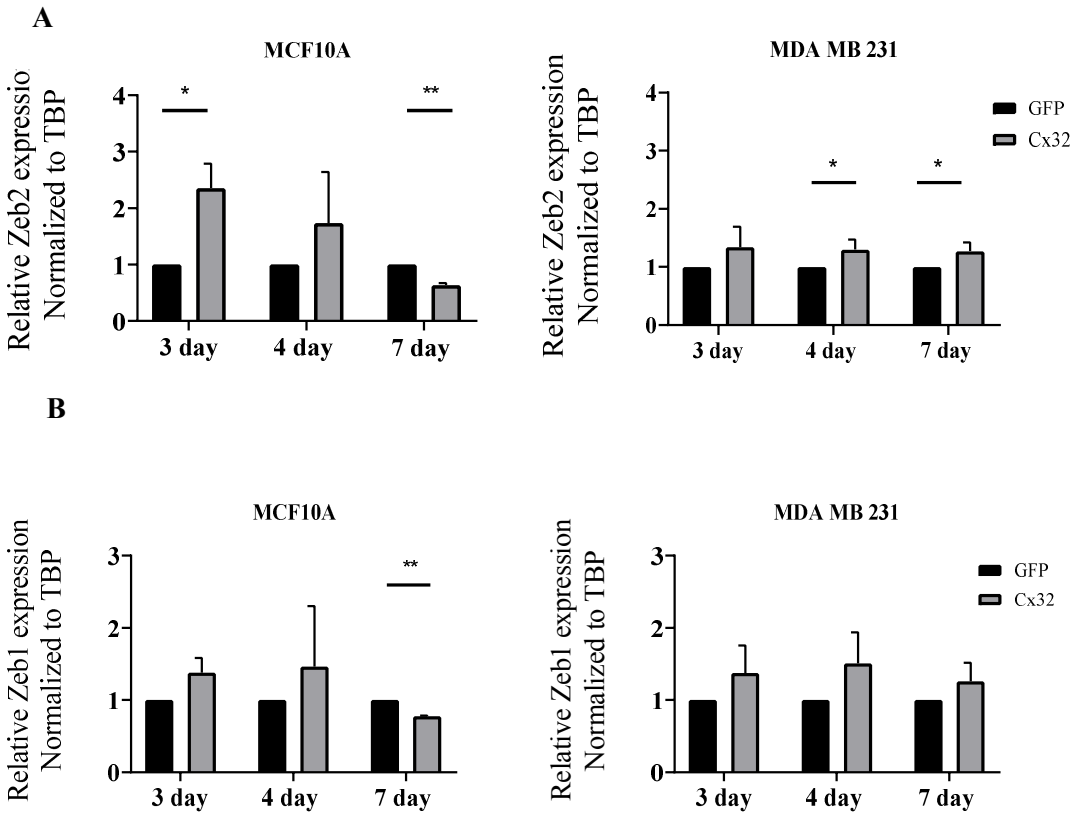


Figure 3.2.13 Relative ZEB family transcription factors expression level both in MCF10A and MDA MB 231 cells. (A) Relative Zeb2 mRNA expression in MCF10A-Cx32 cells with respect to control cells cultured for 3 day (n=2, * p<0,05), 4 day (n=2, ns) and 7 day (n=2, * p<0,05) and in MDA MB 231-Cx32 cells cultured for 3 day (n=3, ns), 4 day (n=3, * p<0,05) and 7 day (n=3, * p<0,05) (B) Relative Zeb1 mRNA expression in MCF10A-Cx32 cells with respect to control cells for 3 day (n=2, ns), 4 day (n=2, ns) and 7 day (n=2, * p<0,05) and in MDA MB 231 cells relative Zeb1 mRNA level expression did not indicate any significant increase for 3 day, 4 day and 7 day cultured cells with respect to control cells. Statistical significance was assessed by unpaired t test and one-way ANOVA and multiple comparison Tukey test (* p<0,05; ** p<0,01; *** p<0,005; **** p< 0,001). The error bars represent the standard deviation.

Moreover, Zeb1 expression indicated significant increase only in 7 day cultured MCF10A-Cx32 cells (Figure 3.2.13B; n=2, ** p<0,01), while it did not indicate any effect in Zeb1 expression of MDA MB 231 cells. In addition; there was not any significant relation between culturing time and ZEB family transcription factors expression (Figure 3.2.13; n=2; ns)

3.2.13 Cx32 overexpression led to changes in Zeb2 expression in time dependent manner for MCF10A cells.

According to the q-RT results, Cx32 overexpression indicated contrary effect on MCF10A cells depending on culturing time (Figure 3.2.13A). To assess whether there is any significant relationship between the culturing time and Zeb2 expression Western blotting was performed both in MCF10A and MDA MB 231 cells.

Results revealed that Cx32 overexpression led to 2,2 (Figure 3.2.14A; n=2, **** p<0,001) fold increase in 3 day cultured MCF10A cells with respect to control cells. However, Cx32 overexpression led to 50% (Figure 3.2.14A; n=2, *** p<0,005) and 28% (Figure 3.2.14A; n=2, ns) decrease in 4 and 7 day cultured MCF10A cells. Furthermore, results revealed that changes in Zeb2 expression is significantly associated with culturing time of the cells.

Unlike to MCF10A cells, Zeb2 expression indicated significant decrease in 3 days cultured MDA MB 231-Cx32 cells (Figure 3.2.14B; n=3, ** p<0,01) followed by 1,85 (Figure 3.2.14B; =3, ns) and 2,17 fold (Figure 3.2.14B; n =3, ns) increase in 4 day and 7 day cultured MDA MB 231-Cx32 cells with respect to control cells.. In addition, no relation was estimated between culturing time and Zeb2 expression in MDA MB 231 cells.

Taken together fold increase in Zeb2 expression indicated correlation with culturing tine of the cells significantly in MCF10A cells which might suggest a relationship between Zeb2 expression and Cx32. That relationship can be based on either change in Cx32 expression level or localization of Cx32 throughout culturing times.

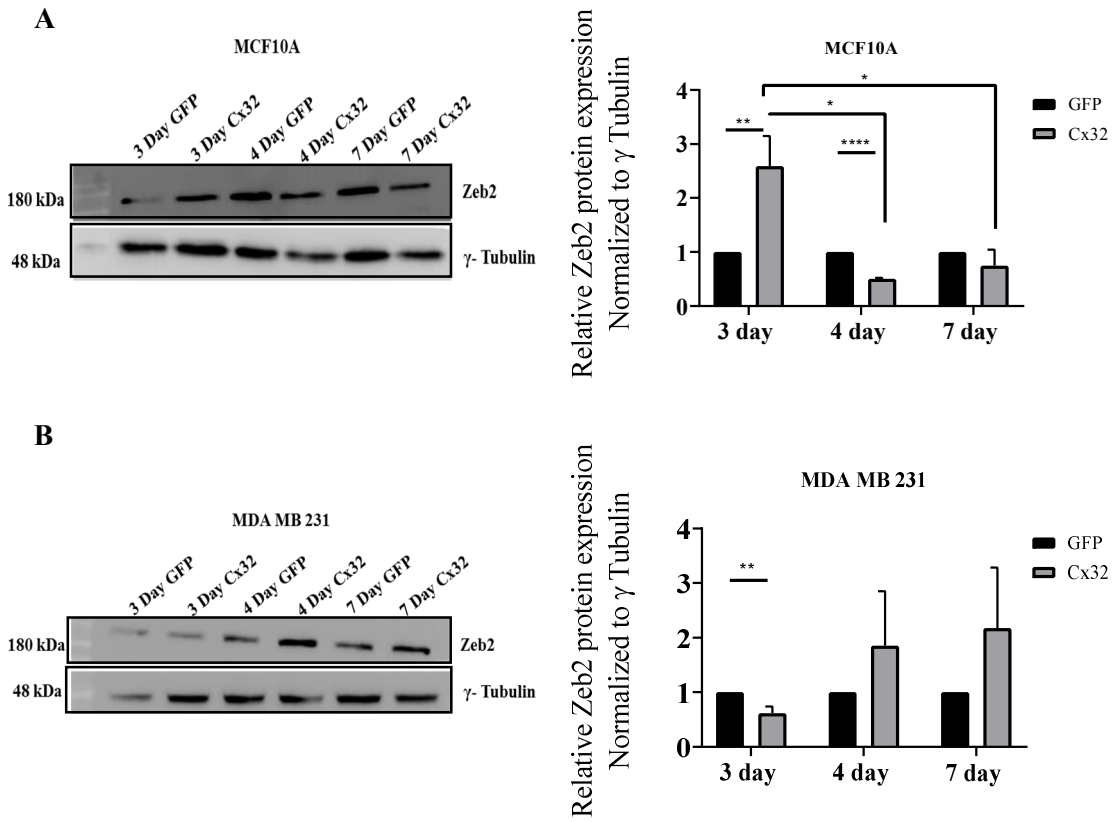


Figure 3.2.14 Zeb2 protein expression level for MCF10A and MDA MB 231 cells. (A) Relative Zeb2 protein expression level for 3 day (n=2, ** p<0,01), 4 day (n=2, **** p<0,001) and 7 day (n=3, ns) cultured MCF10A-Cx32 cells with respect to control cells (B) Relative Zeb2 protein expression level for 3 day (n=3, ** p<0,01), 4 day (n=3, ns) and 7 day (n=3, ns) cultured MDA MB 231-Cx32 cells with respect to control cells. Statistical significance was assessed by un-paired t test and one-way ANOVA and multiple comparison Tukey test (* p<0,05; ** p<0,01; *** p<0,005; **** p< 0,001)). The error bars represent the standard deviation.

Although this correlation was not estimated significantly in MDA MB 231 cells, similar trend between the Zeb2 expression and Cx32 expression throughout different days were noticed. To understand this correlation two-way ANOVA test and Tukey's multiple comparison test was applied and graphs were re-drawn (Figure 3.2.15).

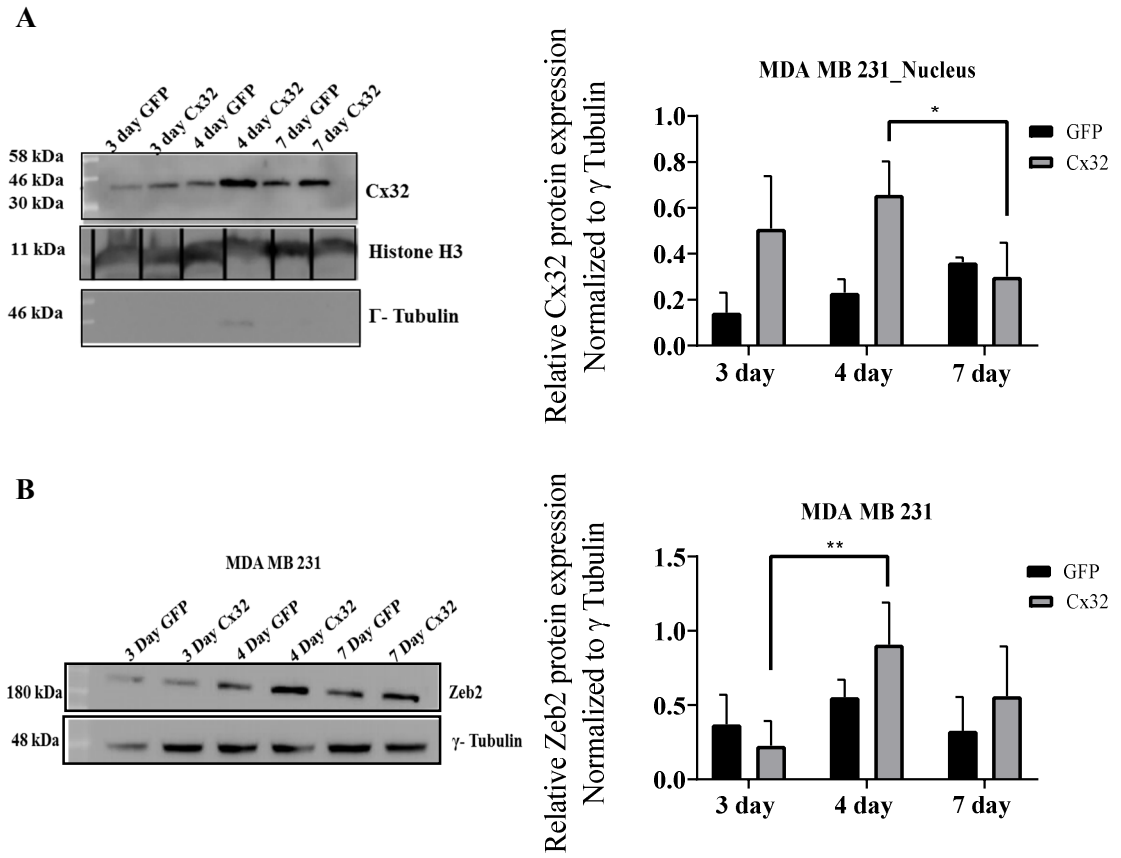


Figure 3.2.15 Zeb2 and nuclear Cx32 level in MDA MB 231 cells. Statistical analysis was performed by two way-ANOVA with Tukey multiple comparison test (* $p<0,05$; ** $p<0,01$)

Results showed similar trend with Cx32 expression and Zeb2 expression which should be enlightened with different molecular analysis in future aspect.

Table 3.2 1 Overall EMT marker changes in protein level both in MCF10A and MDA MB 231 cells.

	MCF10A-3 day	MCF10A-4 day	MCF10A-7 day	MDA MB 231-3 day	MDA MB 231-4 day	MDA MB 231-7 day
Total Cx32	7,4	1,5 (ns)	2,5 (ns)	3,9 (*)	3,9 (*)	⟷
Nuclear Cx32	1,8 (ns)	%50 decrease	2 (ns)	3,9 (**)	3,25 (*)	1,25 (ns)
E-cadherin	⬆	⬆	⬆	⬇	⬇	⬇
Snail	⟷	⟷	⬇	⬇	⬇	⬇
Slug	⟷	⟷	⟷	⬆ in trend	⬆	⟷
Zeb2	⬆	⬇	⟷	⬇	⬆ in trend	⬆ in trend
Vimentin	⬆ in trend	⬆	⬆	⟷	⟷	⟷

Until here, it was suggested that Cx32 indicated time dependent localization especially in MCF10A cells and exogenous Cx32 mostly localized in nucleus in MDA MB 231 cells unlike to MCF10A cells.

In addition, it was found that Cx32 overexpression leads to increase in E-cadherin (epithelial marker) and Vimentin (mesenchymal marker) in MCF10A cells as in a time independent manner. Further, Cx32 overexpression led to decrease in E-cadherin and Snail independent from the culturing time as well (Table 3.2.1). Moreover; Zeb2 indicated different expression trend between MCF10A and MDA MB 231 cells with Cx32 overexpression throughout different culturing times. Importantly Zeb2 expression indicated significant relationship with culturing time in MCF10A cells.

3.2.14 MCF10A cells acquired decrease circularity as independent from culturing time unlike to MDA MB 231 cells.

Next, we asked changes in localization and expression of Cx32 leads to any effect on cellular morphology of MCF10A and MDA MB 231 cells.

In Figure 3.1.4 , it was defined that Cx32 acquired decrease circularity and mesenchymal morphology to MCF10A cells. However, MDA MB 231 cellular morphology did not indicate association with Cx32 overexpression. To understand whether there is a relation with culturing time and cellular morphology, cellular borders of MCF10A and MDA MB 231 cells cultured for 3 days, 4 days and 7 days were defined by phalloidin antibody against actins filaments. Nuclei were probed with DAPI. Morphology of each cell type was deployed by circularity index (complete circle is taken as 1) and analysis was performed in the light of measured circularity index by Image J program. Three images were analysed for each cell type in each set of experiments.

In accordance with Figure 1.3.5 Cx32 overexpression was strongly associated with cellular morphology in MCF10A cells. Here it was shown that Cx32 led to decrease in cell circularity (Figure 3.2.16C; n=3). and acquired mesenchymal phenotype to MCF10A cells (Figure 3.2.16A; n=3). Furthermore, Cx32 effect on cellular morphology did not indicate significant relation with culturing time.

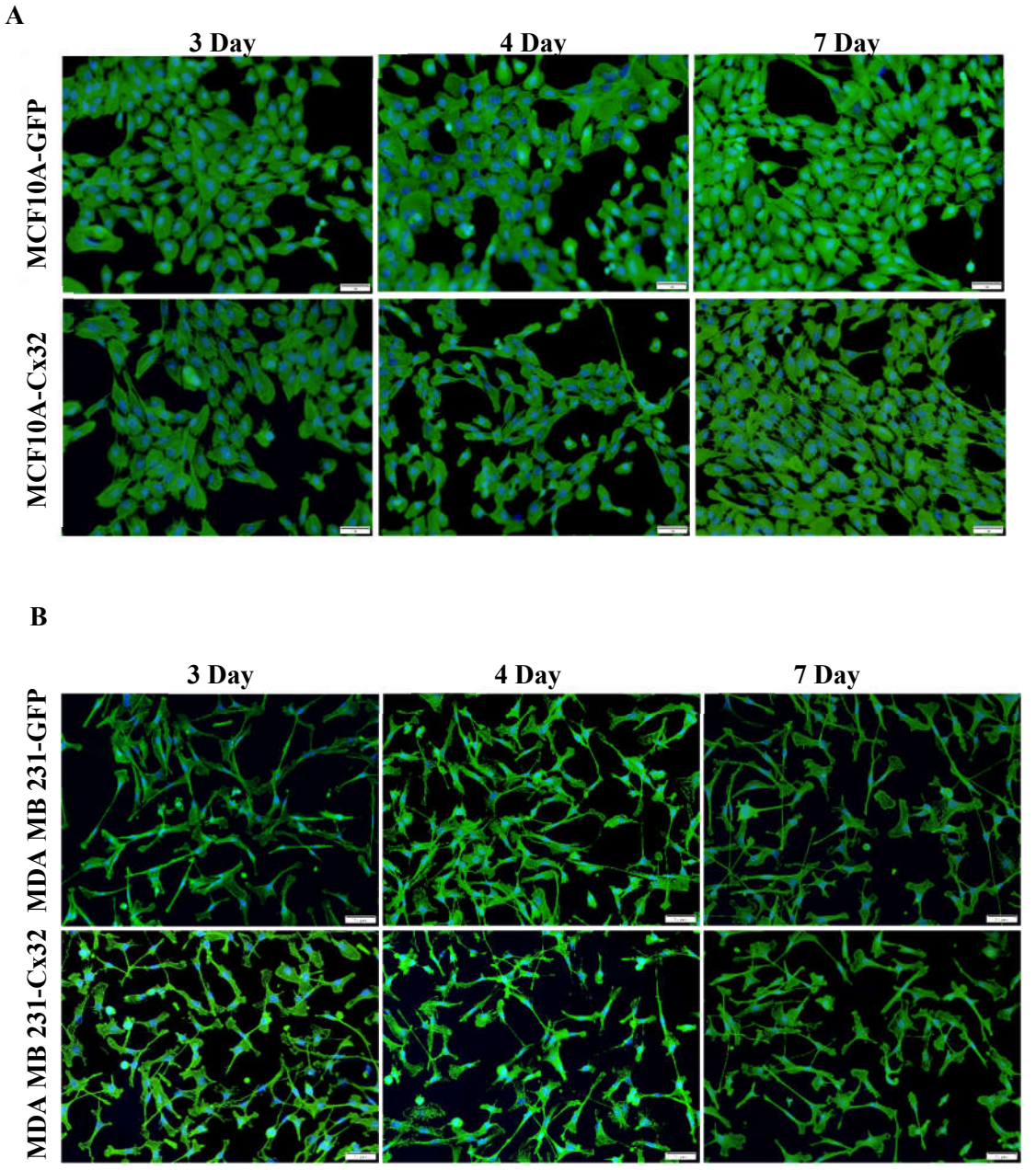

A
3 Day
4 Day
7 Day
MCF10A-GFP
MCF10A-Cx32
B
3 Day
4 Day
7 Day
MDA MB 231-GFP
MDA MB 231-Cx32
C
MCF10A
Circularity
1.0
0.8
0.6
0.4
0.2
0.0
*
**
**
GFP
Cx32
3 day
4 day
7 day
D
MDA MB 231
Circularity
1.0
0.8
0.6
0.4
0.2
0.0
**
GFP
Cx32
3 day
4 day
7 day

Figure 3.2. 16 Visualization and comparison of cellular morphology throughout different time points. Visualization of (A) MCF10A-GFP and MCF10A- Cx32 and (B) MDA MB 231-GFP and MDA MB 231-Cx32 cultured for 3 days, 4 days and 7 days(scale bar =50 μm). (C) Circularity index for MCF10A (n=3, *p<0,05 , ** p<0,01) and (D) MDA MB 231 cells (n=3, ** p<0,01). Statistical analysis was performed by unpaired t test and two way ANOVA with multiple comparison Tukey's test.

Besides, Cx32 overexpression did not lead to changes in cellular morphology for 3 days and 7 days cultured MDA MB 231 cells (Figure 3.2.16D; n=3). Although significant increase was observed in circularity of 4 day cultured MDA MB 231-Cx32 cells, in the light of two way ANOVA analysis not any significant association was found culturing time and morphology with Cx32 overexpression.

CHAPTER 4

DISCUSSION AND CONCLUSION

Our studies revealed cell type and localization dependent role of Cx32 on EMT of breast cancer. In the initial times connexins are mostly considered as tumour suppressors while new developments challenge this generalization and indicated its role in modulation of EMT, metastasis and invasion [138]. In mammary gland, Cx26 and Cx43 are majorly expressed and mostly studied connexin isoforms [139]. In addition to Cx26 and Cx43, Cx32 and Cx30 are other connexin isoforms found in specific stages of mammary gland development [112]. Although the pro-tumorigenic effect of Cx32 on breast cancers was suggested by a clinical report revealed in 2006, until to date the role of Cx32 in breast cancer has not been studied at molecular level [125]. Here, we tried to understand the role of Cx32 in TNBC MDA MB 231 cells with respect to non-tumorigenic immortalized MCF10A cells by concerning different parameters.

To test the Cx32 effect, we selectively overexpressed Cx32 in MDA MB 231 and MCF10A cells. Overexpression of Cx32 led to decrease in proliferation of MCF10A cells by extending the doubling time of the cells but not in MDA MB 231 cells. Large number of observational studies reported connexins' role in proliferation as well [140]. In recent studies, anti-proliferative effect of Cx43 on Hs578T [141], MCF-7 and MDA MB 231 cells [100] and anti- proliferative effect of Cx26 on MCF-7 and MDA MB 435 was indicated [134, 142]. In contrast to these studies, it has been shown that Cx43 enhances the proliferation in Huh7 hepatocellular carcinoma cells, suggesting context dependent effect of connexins [143]. In addition, it has been demonstrated that Cx37 supresses proliferation by arresting rat insulinoma cells in G1 phase while Cx40 and Cx43 do not indicate effect on proliferation in the same type of cells suggesting isoform dependent effect of connexins [144-145]. Furthermore, Li and colleagues reported that cytoplasmic accumulation of Cx32 enhances the proliferation rate in Huh7 hepatocellular carcinoma cells [129]. In accordance with these studies we demonstrated that Cx32 indicated context dependent effect on proliferation and extended the doubling time of MCF10A cells but not that of MDA MB 231 cells. And then we asked how is cell cycle affected from extension of doubling time? To understand that we traced the cells at different time points and observed significant

increases in G1 and S phase of 3 day cultured cells in addition to a significant increase in S phase of 4 day cultured cells (Figure 3.1.3), suggesting Cx32 association with S phase. This situation might be related with checkpoint proteins important for entrance to the S phase or might be related with the completion or exit from the S phase. However, in the literature the association between Cx32 and S phase has not been reported before while its association with G0/G1 arrest through its interaction with Dlgh1 protein in hepatocytes [106] and its association with block in G1 phase through increase in p21 and p27 expression has been reported in gastric cancer cell line [136]. In findings S phase arrest was associated with different checkpoint and signalling proteins. One of them which was also associated with Cx32 is ERK protein. Ming and colleagues suggested continuous activation of ERK protein strongly associated with S phase arrest[146]. However, the effect of ERK activation on cell cycle progression is controversial and Cx32 indirect association with ERK activation was reported and anti-proliferative role of Cx32 has been shown with its negative regulation on ERK activation[147].All in all, we suggested Cx32 strongly involve in proliferation of non-tumorigenic cells and it was probably regulating the S phase. In the light of researches Cx32 might indicate its effect through ERK activation, however, to access certain answers and molecular mechanism behind the Cx32 regulation on proliferation up-regulated and down-regulated genes should be identified between MCF10A and MCF10A-Cx32 cells. S phase is a critical step in cell cycle and multiple checkpoints and multiple complexes are acting a role[148]. Furthermore, candidate proteins should be identified that have possible interaction with Cx32 or indicate any changes by the aspect of either in their expression or post translational modification (activation/ degradation) should be enlightened.

In addition to reduced proliferation, another observable change was noticed in morphological differences of MCF10A cells with Cx32 overexpression. We observed that Cx32 overexpression is associated with more mesenchymal and spindle like shape in MCF10A cells while we did not observe any significant changes in morphology of MDA MB 231 with Cx32 overexpression (Figure 3.1.4). It was previously shown that Cx43 modulates the cellular polarity with regulating the N-cadherin expression level by acting as a transcription factor in the cells. They suggested that Cx43 downregulation led to increase in the cellular circularity and decrease in the cellular polarity of neural crest cells in *Xenopus leavis* embryo [109]. In another study Cx43 downregulation is concluded with alterations in cellular polarity with abnormal front-rear polarity in MCF10A cells [149].

Furthermore, it was revealed that Cx43 did not indicate any morphological effect on TNBC cells as MDA MB 231 and HS578T cells [150]. In the light of findings, acquiring of the mesenchymal phenotype to MCF10A cells with Cx32 overexpression may indicate Cx32 association with EMT.

Up to here we demonstrated that Cx32 overexpression led to decrease in cellular proliferation in MCF10A cells accompanied with more mesenchymal phenotype but not in MDA MB 231 cells. In addition to these studies, we revealed the increased migration capacity in MCF10A cells with Cx32 overexpression (data has been shown in Aslı ADAK thesis project.) but not in MDA MB 231 cells. Taken all, Cx32 overexpression procured mesenchymal characteristics to MCF10A cells but not to MDA MB 231 cells.

In the second part of the study we tried to identify the actors behind this framework and investigated the association between EMT markers and Cx32 expression. In recent studies it has been demonstrated that lacking gap junctional intercellular communication does not mean that cells lack of connexin protein [79]. There is building evidence about the mislocalization of connexins in the cytoplasm. In addition, nuclear connexins have been reported in several reports as well. Kotini and colleagues reported the presence of nuclear targeting signal in C terminal domain of Cx43 facilitating the translocation of Cx43 to nucleus [109]. Moreover, Thiagarajan and colleagues reported localization of Cx26 with NANOG and FAK transcription factors [124]. In addition, aberrant localization of connexins were attributed to the improper trafficking and aberrant phosphorylation of connexins [151]. In parallel with these findings we demonstrated nuclear and cytoplasmic localization of Cx32 both in MCF10A and in MDA MB 231 cells (Figure 3.2.1) and we revealed differential localization of Cx32 through different culturing times.

Before we try to understand the correlation between Cx32 and EMT, first we cultured the cells through different points as 3 days, 4 days and 7 days. In our previous result (data has not shown) we suggested differential localization of Cx32 in MDA MB 231 cells. In accordance with our study, in 2002 differential time-day dependent expression and phosphorylation of Cx43 was reported in rat myocytes [137] that's why we did not perform our experiments based on only one day. In the light of our fluorescent images we revealed majority of Cx32 did not localize in the plasma membrane but localized in the cytoplasm and the nucleus as well. Importantly we suggested that Cx32 localized in nucleus in MDA MB 231 cells throughout all culturing days (Figure 3.2.1B).

However, in MCF10A cell Cx32 localization in nucleus was clearly decreased especially in 4 day and in 7 day cultured cells (Figure 3.2.1A). To confirm these changes, we did subcellular fractionation. Moreover, another parameter about connexins based on their differential expression is different forms (post-translational modifications, truncated forms e.g) at different time points. To understand whether Cx32 indicate differential expression and differential forms between MDA MB 231 and MCF10A cells total protein lysates were probed with Cx32 antibody and differences in Cx32 was revealed between different culturing times as well.

There is a building evidence that dimer formations and different post translational modifications can lead to increase in molecular weight of connexins. In the light of researches Cx32 can form a dimer and can appear in 54 kDa in immunoblot [152]. Ubiquitylation, phosphorylation and N-glycosylation was also reported to lead to appearing of band from 1 to 8-9 kDa above from its actual molecular weight. Besides type of post-translational modifications, their quantity also affects the molecular weight of targeted protein [153]. Our work demonstrated that Cx32 overexpression led to clear increase in band aligned ~46 kDa in MCF10A cells (Figure 3.2.2A)\ while in MDA MB 231 cells (3 day and 4 day cultured cells) Cx32 overexpression led to clear increase in band represents the ~32 kDa band (Figure 3.2.2B). In the light of researches, the band aligned nearly 32 kDa suggesting Cx32 protein, band aligned nearly 46 kDa suggesting Cx32 protein might be the post-translationally modified form. In accordance with these findings Cx32 might be post-translationally modified in both cell type and Cx32 overexpression is associated with increasing in expression of different forms between MCF10A and MDA MB 231 cells (Figure 3.2.2). However, to reach certain prediction, mass spectrometry should be done to determine if Cx32 is post-translationally modified in these cells.

Until here we identified two different parameters about Cx32 between two cells as Cx32 overexpression led to expression of different forms of Cx32 between two cells and Cx32 indicated different nuclear localization pattern between two cells.

Cx32's role in EMT has been demonstrated in several functional studies. Yang and colleagues reported that cytoplasmic Cx32 accumulation associated with increased E-cadherin and decreased Vimentin and Snail expression in HepG2, Huh7 and SMMC-7721 cells [130]. In accordance with previous study Yu and colleagues demonstrated the

strong positive correlation between Cx32 level which is mainly localized in the cytoplasm and E-cadherin level in addition to a negative correlation with Vimentin level in hepatocellular carcinoma cells [132].

We showed that Cx32 led to a significant decrease in E-cadherin (Figure 3.2.5B) level in MDA MB 231 cells while led to a significant increase in E-cadherin level in MCF10A cells. We suggested strong negative correlation between Snail (Figure 3.2.10B) and E-cadherin expression with Cx32 level in MDA MB 231 cells independent of culture time. Snail is a key transcription repressor of E-cadherin and having an important role in metastasis and invasion in breast cancer, while surprisingly here we showed significant decrease both in Snail and E-cadherin expression [61]. Our findings suggest E-cadherin might be repressed in a Snail-independent manner in Cx32 overexpressed cells. In addition to Snail; Twist and Slug are other E-cadherin repressors [37]. Slug demonstrated increasing trend with Cx32 overexpression. Cx32 might indicate its effect on E-cadherin through Slug. However, Slug indicate significant increase only in 4 day cultured cells which might suggest existence of other factors in E-cadherin repression that are activated by Cx32 overexpression. Furthermore, E-cadherin and Snail decrease was observed in both mRNA and protein level suggesting that Cx32 might affect at gene level rather than at protein level such as interference with degradation or protein stability. Moreover, E-cadherin and Snail decrease was observed in all culturing times even in 7 day cultured MDA MB 231 cells where not any significant Cx32 overexpression was seen, Cx32 may indicate long term / epigenetic effect on gene expressions.

Unlike MDA MB 231 cells, in MCF10A cells Cx32 overexpression led to increase in E-cadherin level in culturing time independent manner (Figure 3.2.5A). Anti-proliferative role of E-cadherins have been reported in variety of reports that showed E-cadherin leads to decrease in beta-catenin activity accompanied with G1 arrest [154]. In accordance with this finding we reported G1 arrest in 3-day cultured MCF10A cells (Figure 3.2.3). It has been also reported that E-cadherin can alter the actin cytoskeleton organization and can change the morphology of the cell. However, studies revealed more epithelial hexagonal shaped morphologies with E-cadherin overexpression in zebrafish embryonic cells [155]. In this study we reported mesenchymal phenotype with Cx32 overexpression which might not be correlated with E-cadherin overexpression. In 1998, Handschuh and colleagues reported that if calcium binding motif of E-cadherin is lost or damaged, cell acquired more mesenchymal phenotype [156] that's might be the case why

they have mesenchymal phenotype. In addition, Cx32 is associated with increased Vimentin expression in MCF10A cells (Figure 3.2.8A). In 2010 Vuoriluoto and colleagues reported that Vimentin expression acquired elongated morphology in MCF10A cells with increased migration capacity which has also been shown in our previous study [157].

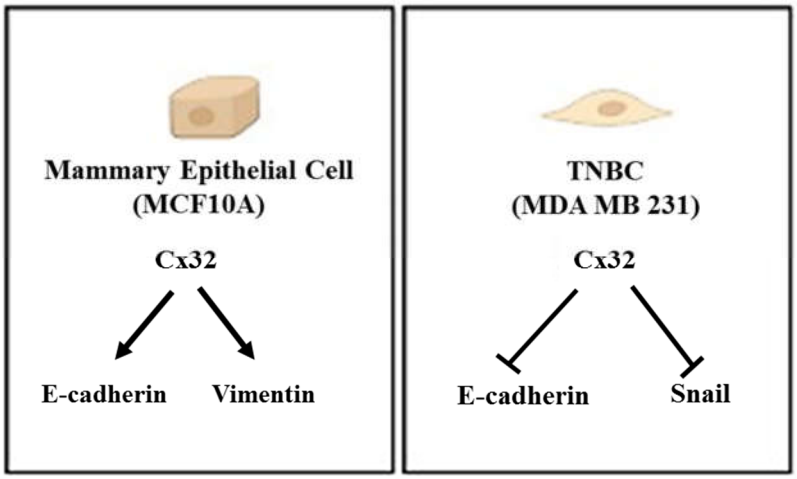


Figure 5. 1 Cx32 overexpression affects the EMT markers A) MCF10A cells B) MDA MB 231 cells.

Until here, we reported consistent changes in EMT markers which did not change according to the culturing time. Taken together (Figure 5.1) , Cx32 overexpression led to increased E-cadherin and Vimentin expression in MCF10A cells while in MDA MB 231 cells Cx32 indicated strong negative correlation between E-cadherin (Figure 3.2.5) and Snail (Figure 3.2.10). In here cell type dependent Cx32 effect might be correlated with differential overexpressed Cx32 forms between MCF10A and MDA MB 231 cells. In addition, Cx32 indicated differential effect on Zeb2 expression between MCF10A (Figure 3.2.13A) and MDA MB 231 (Figure 3.2.13B) cells especially in 3 day cultured cells. Moreover, Zeb2 indicated differential expression pattern throughout culturing times.

Zeb2 is an important EMT modulator that has a positive correlation with metastasis, invasion, proliferation and multidrug resistance in several cancer types [158]. We revealed that Zeb2 expression significantly increased in 3 day cultured MCF10A-Cx32 cells while in 4 day cultured MCF10A-Cx32 cells Zeb2 expression significantly decreased. Differential Zeb2 expression might be related with differential expression of nuclear Cx32 (Figure 3.2.3A) or differential expression of total Cx32 (Figure 3.2.2A) in

MCF10A cells. In accordance with trend, Cx32 protein level decreased in 4 day cultured MCF10A-Cx32 cells when compared to Cx32 level of 3 day cultured MCF10A-Cx32 cells. Moreover, Cx32 localization in nucleus significantly decreased in 4 day cultured MCF10A-Cx32 cells. In here two scenarios can be proposed where Cx32 might need to reach to a certain level to increase the Zeb2 expression or Cx32 might have a direct role in Zeb2 expression (Figure 5.2).

Besides, a similar trend was also observed in MDA MB 231 cells. However,\ unlike MCF10A cells Zeb2 expression indicated decrease in 3 day cultured MDA MB 231-Cx32 cells with respect to control cells. On the other hand, Zeb2 expression had increasing trend in 4 day and 7 day cultured MDA MB 231-Cx32 cells (Figure 3.2.13B). Taken together relation between Zeb2 and Cx32 should be enlightened more with further molecular analysis as Electrophoretic mobility shift assay (EMSA), Chromatin immune precipitation, mass spectrometry or immune precipitation.

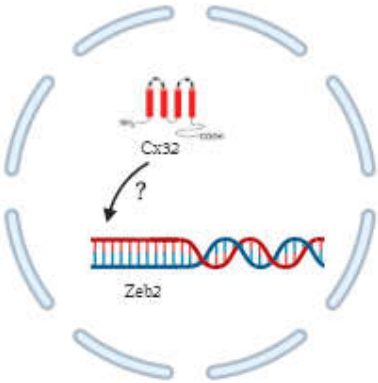


Figure 5. 2 The exact effect of Cx32 on Zeb2 expression should be enlightened with further molecular analysis.

All in all, proteins in our cells are dynamic macromolecules and associated with many different partners which changes according to their localization within the cells as well. Connexins are so complex proteins and can act as a signalling hub and/or transcription factor [109, 124].

Here we investigated the effect of Cx32 on core EMT markers and transcription factors. EMT is a dynamic process, meaning that there is a continuous flux between epithelial and mesenchymal state which facilitates the plasticity to the cancer cells with

motility, stemness, therapy resistance properties [41, 75]. We revealed cell type dependent effect of Cx32 on EMT markers and we suggested importance of localization and form differences as an indicator of connexins' effect. Here we are suggesting differential role of connexin 32 which should be enlightened more with mass spectrometry, immunoprecipitation and chromatin immunoprecipitation assays as well.

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