2014

Barx2 is Sufficient to Drive the Expression of Cadherin-6 in the Developing Nervous System

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Abstract

Barx2 is a homeobox transcription factor linked to cell adhesion, motility, and tumorigenic potential. In the developing chick embryo, the overexpression of Barx2 is used to determine genes responsible for its function. Cadherin-6 (Cad6) is a cell adhesion molecule with similar expression patterns as Barx2 in the renal epithelium, which led us to the idea that a similar association would be found in the central nervous system. Collagen Type II Alpha 1 (Col2A1) encodes for the collagen found in cartilage and Col2A1and Barx2 were indicated to be expressed in the developing neural tube, important for the segmentation of the spinal cord. It was hypothesized that the overexpression of Barx2 would lead to an upregulation of Cad6 and Col2A1 in the developing nervous system, as literature has indicated that in certain non-neural cell types, overexpression of Barx2 induces an upregulated expression of Cad6 and Col2A1. Using immunofluorescence, we determined that overexpression of Barx2 via electroporation in the developing embryonic neural tube promoted expression of Cad-6, but not Col2A1.

Introduction

Barx2 is a homeobox transcription factor involved in the signaling cascades of multiple cell types and is important throughout all development stages. For example, Barx2 is an important factor in the regulation of muscle growth and repair (Meech et al., 2011). Barx2 also shows a cooperative interaction with the transcription factor Sox9 in chondrogenesis during limb development (Meech et al., 2005). Although the role of Barx2 has been investigated in several tissue types, the understanding of Barx2 in the central nervous system is not as well developed. Barx2 expression has been studied in the nervous system of the mouse and the zebrafish model system, but the expression in the developing chick model system has not been analyzed. According to the Allen Brain Institute, mRNA from the Barx2 gene has been detected in the hippocampus of the mouse model, but the protein detection has not been seen in

the developing chick embryo. The expression in the hippocampus may prove to be a potential link to neuroplasticity and memory retention. Barx2 is also highly expressed in neural stem cell populations (Ramalho-Santos et al., 2002), and expression in neural stem cells suggest it may play a role in stem cell differentiation or maintenance (Cambon et al., 2004).

Barx2 has been indicated to serve several roles in driving the expression of cell adhesion molecules (CAMs) in the mouse model system (Sellar et al., 2001). CAMs are a necessary component of tissue development across several cell types because they play a crucial role in cell-to-cell communication and between cells and the extracellular matrix (ECM). Gaining understanding of the regulation and associations among neuronal CAMs and other proteins are of interest because of increase in memory and learning, higher efficacy of synaptic transmission and formation of new synapses, and a potential pathway of neurogenesis (Cambon et al., 2004). An increase in CAMs in the nervous system is beneficial for neural network formation and regulation of synaptic junctions, which are important for memory processes and plasticity (Togashi et al., 2009). We selected the CAM, Cadherin-6 (Cad-6) and focused on the relationship between the overexpression of Barx2 and the resulting expression of Cad-6 in the developing spinal cord of the chick embryo.

Barx2 is also important for chondrogenesis, the development of cartilage. In limb development, precision in the order of signaling and transcription factors is vital for proper development and function of a limb. In this specific cell type, Barx2 regulates gene expression for CAMs, similar to Cad-6, as well as extracellular matrix proteins such as Collagen Type 2 Alpha 1 (Col2A1). Col2A1 is the gene that encodes for a fibrous protein component of cartilage (Meech et al., 2005). But this is in limbs and mesenchymal tissue, and we wanted to determine if this signaling pathway applies to tissue in the nervous system. When studied in the zebrafish, Col2A1 is expressed in the developing spinal cord in the dorsal neural tube, as well as the neural crest (Yan et al., 2005). It is these extensions of the role of Col2A1 that allowed a hypothesis to be drawn: If Barx2 drives the expression of Cad-6 and Col2A1 in these tissue

types and these factors are located in the same cells in the nervous system, then Barx2 may drive expression of Cad-6 and Col2A1 in the nervous system. To test this, we overexpressed Barx2 in the developing chick embryo to determine if it was sufficient to lead to an upregulation of Cad-6 and Col2a1 expression

In ovo electroporation with Barx2 vectors that contain a reporter protein, EGFP, drives the overexpression of Barx2 in successfully transfected cells. Immunofluorescence can then be used to detect the protein overexpression of Barx2, and can determine if more cells are expressing Cad-6 and Col2A1. Electroporation uses a positively charged cathode to pull negatively charged DNA into developing tissue, driving the overexpression. Immunofluorescence uses a known antibody to bind to a protein that is hypothesized to be present. Adding a fluorescent protein, EGFP, allows for the detection and identification of the protein, Barx2. Cad-6 and Col2A1 protein expression was identified using a red fluorescence.

Methods

Chickens

Fertilized White Leghorn chicken eggs were obtained from the Michigan State University Poultry Research and Teaching Center in Lansing, Michigan. The eggs were stored at 13˚C; when they were needed for an experiment they were brought to room temperature and then incubated at 38°C for 44 hours prior to electroporation.

Transfection by Electroporation

DNA Precipitation

Barx2 pCIG expression vector was generated by previous students within the lab (Russo et al., unpublished) Barx2 and pBluescript (PBS) or pCIG (the empty vector) and pBluescript were combined

and precipitated for a final concentration of 3 μg/μL, specifically 0.5 μg of Barx2 or pCIG, and 2.5 μg of PBS. These volumes were combined and a 10% volume of 3M sodium acetate was added. After vortexing briefly, 2 volumes of 100% ice cold ethanol were added and the solution was spun down at maximum speed for 2 minutes. The supernatant was removed carefully and the pellet was rinsed with 200 μL of ice cold 70% ethanol 3 times, then the pellet was air dried. Once all the ethanol had evaporated, the pellet was re-suspended in 10 μL of 18 MΩ H_2O. The DNA was transferred to a PCR tube and fast green was added as a visual aid during electroporation. The same procedure was repeated for both sets of DNA; Barx2 + PBS and pCIG + PBS.

In Ovo Electroporation

A 1X volume of penicillin-streptomycin was added to 1X Tyrode's Saline, without calcium or magnesium (CMF), and the india ink was prepared using Pelikan India Ink (Germany) and CMF. Embryos were retrieved from the incubator 3-6 at a time after 44 hours of incubation, and were taped with scotch tape, stabilizing the shell and allowing a window to be cut to allow access to the embryo. A 10 mL syringe with a 16 gauge needle was used to remove 5-7 mL of albumin. The window was cut using surgical scissors and any waste was dispensed into a biohazard bag. A 3 mL syringe with a 26 gauge needle was used to inject approximately 50-100 μL of india ink under the embryo to create contrast in order to visualize the embryo. A pulled glass capillary tube was trimmed, and used for DNA injection into the developing spinal cord. The trimmed capillary was guided into the top of the brain, into the developing spinal cord, and DNA was injected, dispersing into the spinal cord. 1 mL of the CMF solution was added to the embryo and the electrodes were placed 4 mm on each side from the embryo. The electrical impulse was applied at an electric current of 18 V, and the negative DNA is drawn towards the positive cathode. About 1 mL of CMF was added to the embryo to bring down the temperature and the window was replaced and sealed with parafilm. The eggs were placed back into the incubator, which

was filled with tap water to insure a humid environment to prevent the eggs from drying out. The electroporated embryos incubated for 36 hours.

Harvesting

The embryos were harvested 36 hours and those positive for Barx2 overexpression in the spinal cord (detected under the fluorescence of the dissecting microscope) were preserved. The embryos were fixed for 45 minutes in 4% paraformaldehyde (PFA) at 4°C, washed in 0.1M phosphate buffered saline (PBS), and cryoprotected overnight at 4°C in 15% sucrose. The tissues were then mounted in Tissue Tek OCT (VWR, West Chester, PA). They were frozen using liquid nitrogen, and sectioned into 12-micron sections using a Leica cryostat. These sections were then mounted on glass microscope slides. These slides were stored at -20°C until they were retrieved for immunofluorescence.

Immunofluorescence

Staining was performed using 36 hour tissue sections to detect protein expression of Barx2, Cad-6 and Col2A1. The slides were retrieved from the -20°C and thawed in 1X PB. The slides were placed in the staining vessel and blocked with GSS (5% goat serum, 0.1% Triton X 100 in a 50 mL total volume). For staining for Barx2 and Cad-6, the Cad-6 antibody (Mouse IgG1, DSHB #CCD6B-1) and EGFP (rabbit polyclonal EGFP, Abcam) were applied. For Barx2 and Col2A1, the Col2A1 antibody (Mouse IgG1, DSHB #CIIC1) and EGFP (rabbit polyclonal EGFP, Abcam) were applied. The Cad-6/Barx2 solution was added to one slide, and the Col2A1/Barx2 solution was added to the other slide. The vessel was filled with 3 mL of water to prevent the slides from drying out and the lid was placed and sealed with parafilm. The vessel was stored overnight at 4°C. After 12-16 hours the slides were washed in 1X PB 3 times at 10 minute intervals. Secondary antibody solutions were added to appropriate slides. For Cad-6/Barx2, the secondary antibodies were 1:1000 AF555 IgG1 (Invitrogen), and 1:500 FITC goat anti-rabbit (Jackson Laboratories). For Col2A1/Barx2, the secondary antibodies were 1:1000 AF555 IgG1, and 1:500 FITC goat anti-rabbit

(Jackson Laboratories), and counterstained with DAPI (Sigma; 1 mg/ml) at a 1:500 concentration to visualize the nuclei of individual cells. The slides were incubated at room temperature for 30 minutes. After 3 more washes in 1X PB for 10 minutes each, and the slides were washed with 4% PFA. The coverslips were set with Mowioli (Fluka, #81381, fluorescence preservative). The slides were dried at room temperature for at least 24 hours and were then monitored for expression under fluorescence using the Olympus Bx350.

Results

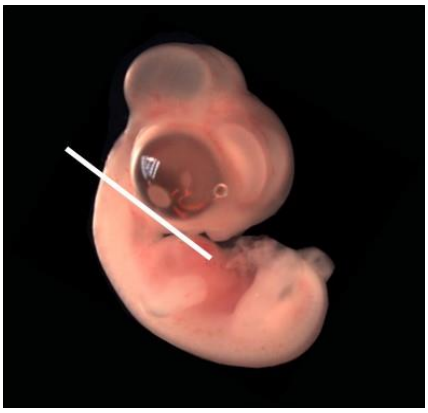

Figure 1. Sagittal view of 120 hour chick embryo. This figure is a representation of sectioning. The white line represents the position of the blade as it sections through the spinal cord. The sections are taken at multiple axial levels.

To overexpress Barx2 and determine if it regulates the expression of Cad-6 and Col2A1, chick embryos were transfected using in ovo electroporation. Immunofluorescence was used for the detection of the protein expression of Cad-6, Col2A1, and Barx2. Cross-sections of the spinal cord were taken after the chick embryo incubated for 36 hours. Several sections of the spinal cord were on the same slide, as serial sections, allowing expression to be monitored throughout the spinal cord. Cross sections are obtained by applying the blade perpendicularly to the spinal cord (Figure 1). These sections were taken at a 12 micron thickness.

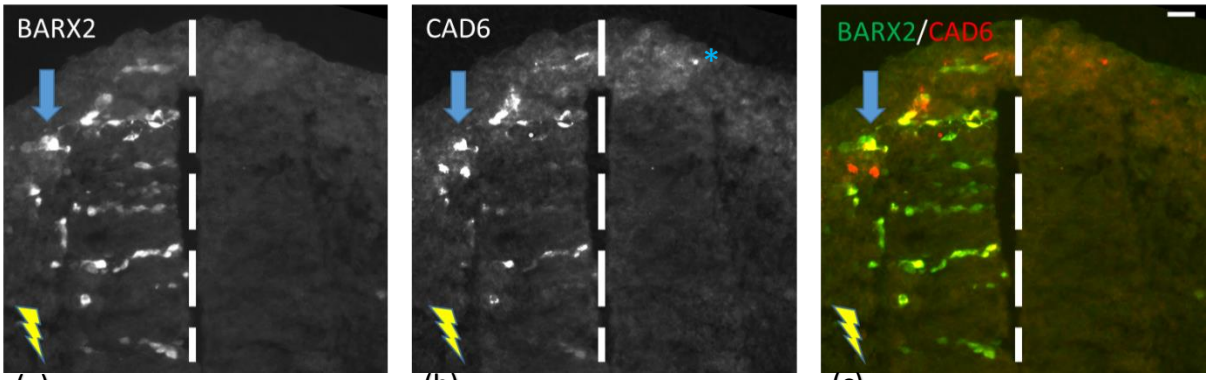

Figure 2. Cross-section immunofluorescence of chick embryo from 36 hr time point of incubation. These immunofluorescence images are gathered from staining for the Barx2 and Cad-6 protein expression with antibodies. Barx2 expression (a), Cad-6 expression (b) and the overlay of images (a) and (b) (c).

As a consequence of the electroporation process, one half of the neural tube is transfected and overexpresses Barx2, and the other half serves as an internal control of expression. For this representation, the electroporated side is identified using a lightning bolt and to indicate the experiment (Barx2 overexpression) side from the control, the midline is created by a dashed line (Figure 2). Barx2 was overexpressed and cells that expressed Cadherin-6 and Collagen Type 2 Alpha 1 were compared to the cells that overexpressed Barx2, both in location and the number of cells in order to draw conclusions about how these genes are influenced by the overexpression of Barx2.

For this experiment, antibodies to detect Cad-6 expression and Barx2 overexpression were applied to the sections, and fluorescence from their respective secondary antibodies was detected using different filters. One filter detects the EGFP of Barx2 overexpression (Figure 2a), and another is used to detect the red fluorescence of the Cad-6 stain (Figure 2b). Adobe Photoshop was then used in order to create an overlay in order to determine coexpression (Figure 2c). The Barx2 overexpression is displayed in black and white fluorescence in order to focus on the cells that were overexpressing Barx2 versus cells that are not overexpressing Barx2 (Figure 2a). Barx2 overexpression is present in multiple cells, extending medially to laterally, and immunofluorescence indicates that the intensity of Barx2 staining was stronger in some cells than others, indicating that expression levels of Barx2 varied in different cells (Figure 2a). There is no fluorescence to the right of the midline, which represents the internal negative control. A cell distinctly expressing Barx2 is identified by an arrow (Figure 2a). The expression for Cadherin-6 was also monitored and displayed as a black and white fluorescence (Figure 2b). The cell clearly expressing Barx2 in Figure 2a is also indicated in Figure 2b with an arrow. One cell with Cad-6 endogenous expression on the control side of the experiment is indicated by the blue asterisk (Figure

2b). The final composite was an overlay of Barx2 and Cadherin-6 (Figure 2c). In Figure 2, the overexpression of Barx2 is green, the expression of Cadherin-6 is represented with red, and the cell, identified with a box, in yellow illustrates the coexpression of Barx2 and Cadherin-6 (Figure 2c). Some red fluorescence is detectable at the neural crest on the right side of the midline, which is squared for clarification (Figure 2c).

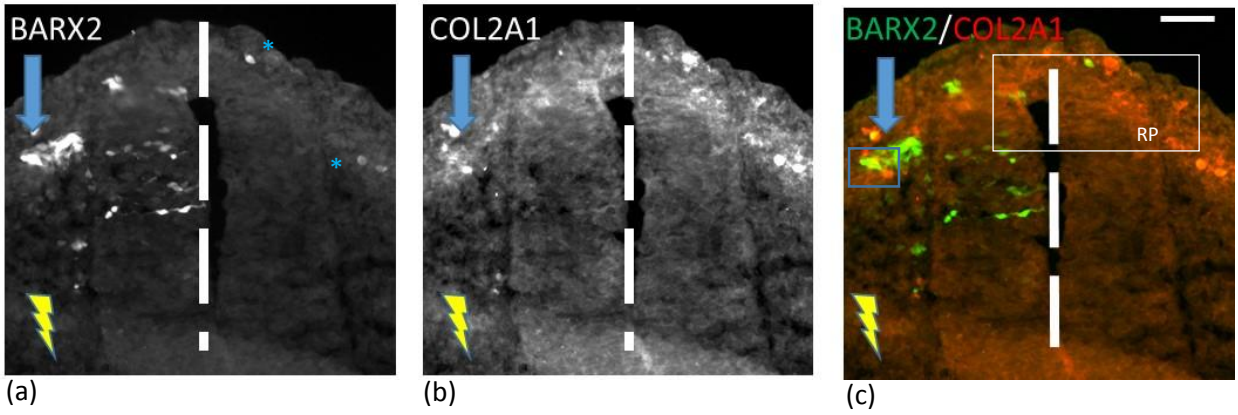

Figure 3. Cross-section immunofluorescence of chick embryo from 36 hr time point of incubation. These immunofluorescence images are gathered from staining for the Barx2 and Col2A1 protein expression with antibodies. Barx2 expression (a), Col2A1 expression (b) and the overlay of images (a) and (b) (c).

As the immunofluorescence was applied for Cad-6 and Barx2, the same was applied for Col2A1 and Barx2, using the same methods but with the Col2A1 primary and secondary antibodies. The fluorescence were separated EGFP for Barx2 and red for Col2A1, and overlayed using Adobe photoshop to determine cells that have coexpression of Barx2 and Col2A1. Similarly to the stain involving Barx2 and Cadherin-6, Barx2 overexpression was presented as a black and white image and cells overexpressing the protein extend from medially to laterally on the electroporated side (Figure 3a). A few cells, marked with an asterisk, have visible fluorescence on the un-electroporated side of the cross-section, indicating endogenous expression (Figure 3a). A specific cluster of Barx2 overexpressing cells has been identified by an arrow (Figure 3a). The stain for Col2A1 shows expression on both sides of the midline, which is

symmetrically localized to the neural crest (Figure 3b). An arrow indicates an example of Barx2 overexpressing cells that are expressing Col2A1 (Figure 3b). For the overlay, the same cluster of cells is being identified using an arrow, as well as the color overlay of Barx2 (green) and Col2A1 (red) and coexpression of proteins (yellow) (Figure 3c). The cross-section has a red tint to it, on top of the cells that are expressing Col2A1 and showing red (Figure 3c). In the indicated group of cells, a few are expressing Barx2 and a few are expressing Col2A1, but it only shows one cell expressing both (Figure 3c). The cells positive for Col2A1 expression are likely part of the somites, which can stain positive for Col2a1 at this stage in mice and can also stain some of the ectoderminal tissue (indicated with arrowheads).

Discussion

Our hypothesis was that the overexpression of Barx2 would up-regulate the expression of Cad-6 and Col2A1 in the developing nervous system of the chick, based on the action of Barx2 in other non-neural cell types. Overexpression of Barx2 was manipulated using the technique of electroporation. The electroporation experiment used Barx2 vectors that co-expressed the fluorescent reporter protein EGFP, as well as vectors that contain the fluorescent reporter gene only. An immunofluorescence experiment, using known antibodies to detect proteins, were used to determine a successful overexpression of Barx2, and its ability to regulate Cad-6 and Col2A1 expression. The immunofluorescence results of Cad-6 and Col2A1 were compared to the protein expression results of Barx2, both in localization of expression, and quantity of cells expressing the proteins. Successful overexpression of Barx2 was shown by inspecting a whole embryo using the dissection scope and the fluorescence of EGFP. Embryos with strong expression of Barx2 were sectioned and analyzed by immunofluorescence. We stained tissue sections using immunofluorescence to determine if Barx2 overexpression would increase the expression of Cad6 or Col2a1.

To test for the effect on Cad-6 regulation by Barx2 overexpression, we used immunofluorescence to identify cells coexpressing Barx2 and Cad-6, from tissue sections stained with the Barx2 and Cad-6 antibodies. Barx2 overexpressing cells are easily distinguishable in tissue sections. When the same section was stained with the antibody for Cad-6, we found that Cad-6 is localized predominately in Barx2 overexpressing cells. The arrow indicates one example of a specific cell that expresses both Barx2 and Cad-6 (Figure 2c).

Endogenous expression of Cad-6 was also detected in these sections of the spinal cord. For example, cells that expressed Cad-6 but did not show Barx2 overexpression were detected on both sides of the neural tube, the treated side and the control side. Endogenous expression of Cad-6 in these cells is assumed because we know that it was not linked to the overexpression of Barx2, because for the Barx2 results, no cells were identified on the control side for overexpression. The overlay for coexpression of Barx2 and Cad-6 supports the conclusion of the relationship between Barx2 and Cad-6. The same cells (reference arrow in Figure 2) that had indicated the cell expressing Barx2 and Cad-6 are yellow in the overlay, demonstrating coexpression of Barx2 and Cad-6. Coexpression of Barx2 and Cad-6, as indicated by Figure 2c (see arrow), supports the conclusion that the overexpression of Barx2 leads to an upregulation of Cad-6 in the central nervous system. This upregulation and association of Barx2 and Cad-6 are also supported by the higher number of cells expressing Cad-6 on the experimental side, compared to the control side (Figure 2b). These observations attest to Barx2 overexpression leading to ectopic expression of Cad-6 in the cells that overexpress Barx2, all of which is consistent with observations in the literature about Barx2 overexpression upregulating Cad-6. This suggests that there is physiological context for their association.

Cad-6, as a cell adhesion molecule, allows cells the capability for cell-to-cell adhesion, and is necessary for normal tissue development, and the relationship with Barx2 has only been detected in tissues outside of the nervous system in other models (Meech, 2005). For example, in the mouse, Barx2

is shown to induce the expression of Cad-6 in ovarian cancer cell lines (Sellar, 2004). Induced expression of an adhesion protein in a cancer line might lead to the conclusion of a more aggressive, metastatic tumor with a faster growth rate (Stevens, 2006), but in this line, Barx2 induction of Cad-6 is a suppressor of tumor progression (Sellar, 2004). Establishing a relationship between Barx2, a transcription factor shown to affect regulation of cellular adhesion molecules is novel in the nervous system, because while the presence of mRNA for Barx2 and Cad-6 have been seen in the hippocampus (according to in situ hybridization images provided by the Allen Brain Institute), regulation of Cad-6 by Barx2 has not been shown until this study.

When immunofluorescence was used for Col2A1 expression, the Barx2 and Col2A1 antibodies were applied to the sections of tissue. Again, there is a clear distinction of cells overexpressing Barx2 on the experimental side. Barx2 overexpression in the Col2A1 stain was localized similarly to the Barx2 overexpression section for the Cad-6 stain (compare Figure 2a, Figure 3a). When the sections were stained using the Col2A1 antibody, we found that there was no preferential expression of Col2a1 with Barx2 overexpressing cells. Col2A1 expression was symmetrical between the treated and untreated sides of the neural tube (Figure 3b, 3c), and when comparing with Barx2 overexpression, it is noticeable that the expression of Col2A1 is independent of the overexpression of Barx2. Interestingly, the arrow (Figure 3b) indicates a specific cell where Col2A1 expression is present when Barx2 overexpression is present, suggesting that Barx2 does not suppress Col2A1. This is likely due to endogenous expression of Col2A1. Indeed, the overlay, Figure 3c, demonstrates the expression of Col2A1 is on the experiment and control side, which is significant with the endogenous expression of Col2A1, consistent with observations from the literature (Yan et al., 2005). There is one cell shown to be coexpressing Barx2 and Col2A1 (see arrow in Figure 3c). This may be explained by a cell that endogenously expresses Col2A1 that incorporated Barx2 overexpression as it migrated from the midline to the neural crest. Thus these data indicate there were not enough cells coexpressing Barx2 and Col2A1 to confirm a regulation effect

of overexpressing Barx2 for Col2A1. Barx2 overexpression, while indicated to drive expression of Col2A1 in other non-neural tissues (Meech et al., 2005), doesn't appear to drive its expression in the early embryonic nervous system under these conditions.

The hypothesis that Barx2 could regulate Col2a1 expression stemmed from the role of Barx2 expression on Col2A1 in chondrogenesis. During limb development, Barx2 regulates Col2A1 in the limb bud (Meech et al., 2011). We wondered if Barx2 overexpression would effect Col2A1 expression in the nervous system, because in the zebrafish, Col2A1 is a determining factor in the embryonic axis of the developing nervous system. Exploring the relationship of Barx2 and Col2A1 has never been done in the developing nervous system, and our chick model system gave us the opportunity to determine if it was possible.

The regulation of these genes by Barx2 were studied in the normal developing nervous system, but it is also important to understand gene expression in pathophysiology. Pathophysiology is the pathway in a disease state or cell line, such as cancer. Focusing on the relationship of Barx2 in a pathophysiology directs us to two other genes as future directions: the estrogen receptor (ER) and matrix metalloproteinase 9 (MMP9). The estrogen receptor, present on cells and responsible for responding to estrogen in the system, is necessary for development. In a pathophysiology cell line, such as in renal cancer, increased ER can lead to the formation of anchorage- independent growth in tumors [cite]. This allows tumors to form without being attached to tissue. It is interesting how Barx2 with Cad-6 can suppress tumor growth, but when ER is involved, it may lead to promotion of tumor growth. Matrix metallo-proteinases are enzymes that digest the extracellular matrix, a necessary part of duct formation. Unfortunately, in pathophysiology, an uncontrolled increase of these enzymes can be associated with more aggressive metastatic capabilities of cancer as they allow cancer cells to migrate through the extracellular matrix. Further work will determine genes regulated by Barx2 in the nervous system and its relationship to normal physiological function as well as pathophysiology.

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