RUNX2 IN EMBRYONIC HEART DEVELOPMENT AND HEART DISEASE

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ABSTRACT

Runx2 is a transcription factor with well-documented roles in skeletal development and bone formation. Runx2 expresses two isoforms which differ in only one exon at the 5' region. Our research has shown that only one isoform of Runx2, Runx2 (I), is expressed in the embryonic chicken heart and has a function in the activation step of epithelial-to-mesenchymal transition (EMT). Observations made using Runx2 siRNA show that knocking down Runx2 (I) also down-regulates EMT-related transcription factors Snail1, Twist1, and Zeb1 and also decreases the expression of mesenchymal markers. Because of Runx2's known role in the bone, we also observed its function during the calcification of the heart. Using AVM cells grown using the micromass tissue culture technique, we observed that the Notch signaling pathway may be most important to the regulation of Runx2 isoforms during this process. We also observe that during calcification Runx2 (II) expression is significantly up-regulated, suggesting differential functions of the two isoforms. By observing the functions of Runx2 isoforms in the heart, we hope to shed light on this transcription factor's function in other tissues, as well as the specific roles of each isoform in the heart.

INTRODUCTION

Heart disease is the leading cause of death in the United States, with calcific aortic stenosis the third leading cause of cardiovascular disease, behind hypertension and coronary artery disease (Garg, 2006 and Yang et al., 2009). It is estimated that about 2-3% of the population is affected by calcific aortic stenosis by 65 years of age. Originally it was thought that aortic valve calcification was a degenerative process associated with aging, but growing evidence suggests that this calcification may be an active disease state resulting from dysfunctional molecular and genetic pathways (Garg, 2006). It's been hypothesized that this calcification process in the heart is similar to that of active bone formation (Garg, 2006 and Yang et al., 2009).

Runx2 is a transcription factor with well documented roles in skeletal formation as a regulator of bone genes (Mendoza-Villanueva, et al., 2010; Pratap, et al., 2006 and Shore, 2005). It has also been shown to have roles in normal mammary epithelial cell function. Recently, Runx2 is shown to be over-expressed in breast cancer cell lines that metastasize to the bone, allowing metastatic cancer cells to form osteolytic bone lesions (Mendoza-Villanueva, et al., 2010; Pratap, et al., 2006 and Shore 2005). Not surprisingly, Runx2 has also been found to be up-regulated in calcified aortic valves as well (Garg, 2006 and Yang, et al., 2009). This finding prompted our interest in Runx2 and its possible functions in the developing heart, as well as the calcification process.

Proper skeletal development requires the coordination of multiple signaling pathways which control the amount of Runx2 expressed. BMP is a potent osteogenic protein that is required for osteoblast differentiation and bone formation. It has also been shown to be implicated in vascular calcification (Li et al., 2008 and Yang, et al., 2009). It was shown recently though that Runx2

expression is induced by BMP-2 signaling, which in turn inhibits the Notch1 pathway (Ann, et al., 2010; Cohen, 2010; Li, et al., 2008; Pratap, et al., 2006 and Yang, et al., 2009). Notch1 was shown to control osteoblast differentiation by repressing Runx2. Accumulation of Runx2 though, suppresses Notch1 signaling by a direct physical interaction (Ann, et al., 2010). Smad1 has also been shown to be required for the expression of Runx2, after BMP-induced signaling (Yang, et al., 2009). Runx2 activity is also regulated by an important binding partner, Cbfβ, which appears to regulate DNA binding (Mendoza-Villanueva, et al., 2010).

Runx transcription factors are defined by the presence of a conserved DNA binding domain, called the Runt domain. The Runt domain interacts with Cbfβ, a transcriptional co-activator which is recruited to the promoters of the Runx family of proteins. Cbfβ binds to the non-DNA binding surface of the Runt domain and causes structural changes in the DNA-recognition surface, increasing its affinity for DNA (Cohen, 2009; Kanatani, et al., 2006 and Mendoza-Villanueva, et al., 2010). Cbfβ itself does not come into contact with the DNA. The Runx2 gene has two promoters resulting in the expression of two isoforms differing in their N-termini; a long form known as Runx2-II and a shorter form known as Runx2-I (Cohen 2009; Kanatani, et al., 2006 and Stock and Otto 2005).

Both isoforms are expressed in osteoblasts and chondrocytes, but Runx2-II is also expressed in osteoprogenitors and other mesenchymal tissues. Research shows that the isoforms keep osteoblasts in an immature stage and inhibit the transition of osteoblasts to osteocytes thereby maintaining the supply of immature osteoblasts for bone formation (Cohen, 2009; Kanatani, et al., 2006; Komori, et al., 2006 and Stock and Otto 2005). The two isoforms have also been shown to be differentially expressed and display their own unique functions. For example, Runx2-I is expressed in the embryonic heart (data below), while Runx2-II is primarily localized

to the bone. Runx2 expression may account for the observed calcification of heart valves. A possible cause of calcification may have to do with the incorrect isoform being expressed and up-regulated in the heart. Runx2-I is also associated more with metastatic cancer phenotypes, as it has a role in epithelial-to-mesenchymal transition (EMT) (see below). Both variants require Cbfβ to enhance their activity, but evidence shows that Cbfβ may be regulating each isoform differently causing differential expression of the two isoforms (Kanatani, et al., 2006).

Twist1, another transcription factor involved in bone differentiation and also EMT, regulates the activity of Runx2 by having an inhibitory effect (Komori, 2006). There is also data to suggest though that Twist1 can up-regulate Runx2 expression in different cell lines. This contradictory evidence suggests that Twist1 most likely interacts with other proteins to modify Runx2 expression (Yuen, et al., 2008). Snail1 and Snail2, transcription factors with well-documented roles in EMT, are negative and positive regulators, respectively, of Runx2 (Lambertini, et al., 2009; Romano and Runyan, 1999 and Park, et al., 2010). Snail2, which is positively correlated with osteoblast markers, was shown previously in the lab to be regulated by TGFβ2 in accordance with the activation step of EMT (Lambertini, et al., 2009 and Romano and Runyan 1999).

Epithelial-mesenchymal cell transition (EMT) in the embryonic chick heart is a regulated process where endothelial cells lining the atrioventricular (AV) canal transform and invade the underlying matrix to form valve progenitors (Runyan, et al., 1983). This EMT is mediated, in part, by the sequential activities of TGFβ2 and TGFβ3 in steps of activation and invasion, respectively. Because of observations suggesting Runx2 is expressed in the early atrioventricular canal, we also decided to investigate Runx2's role in EMT (Tavares, unpublished data). An

initial screen using siRNA against Runx2 suggested that it was functional during the EMT process, prompting us to explore the molecule further.

While a large amount of research exists for Runx2's function in bone calcification, its role in the heart in regards to aortic valve calcification is not well understood. Deducing the roles of each isoform in this process will lead to a better understanding of not only heart disease, but of cancer as well, as breast cancers preferentially metastasize to the bone. By examining the role of Runx2 in embryonic heart development and the calcification process, we hope to elucidate the specific functions of each isoform in the heart.

MATERIALS AND METHODS

Heart Explant Procedure

Chicken eggs were obtained from McIntyre Poultry and Fertile Eggs in Lakeside, California. Fertilized eggs were incubated for 50-58 hours at 37.5ºC to obtain Stage 14 and 16 embryos (Hamburger and Hamilton, 1951). Embryos were removed from the yolk and washed thoroughly in Sigma's Tyrodes solution and then staged (Runyan, et al., 1983). The AV canal was then removed to be used in further experiments.

Collagen Gel Preparation

Rat-tail collagen was first mixed in a 2:1 ratio with distilled water and labeled tube 'A'. Another tube 'B' was prepared with a 1:1 ratio of 2.2% sodium bicarbonate and Sigma 10x Media 199. Next tube A and tube B were mixed in a 4:1 ratio in a new tube, 'C'. After vortexing, the solution was pipetted into a four well plate in 300uL aliquots. Gels solidified at 37ͦC for 30 minutes. After gels had solidified, 300uL of Complete Media 199 was added to each well and incubated for at least two hours. Complete Media 199 contains M199 1X, 100X of insulin transferase selenium (ITS), fungisome (PSF) and chicken serum (Runyan, et al., 1983).

Micromass Tissue Culture Technique

Atrioventricular mouse (AVM) cells were plated at a cell density of 10,000 cells/uL at the center of 35mm plates. Previous studies by Solursh and colleagues showed that embryonic limb tissues would form calcified cartilage masses when cultured in high-density culture (Ahrens, et al., 1977). We found that cardiac cushion cells, but not a mouse L cell fibroblast line, similarly formed calcified tissue when cultured at a similar density. This observation was exploited to explore calcification by cushion tissue cells.

Cells were seeded in 100uL drops overnight. The next day 2mL of 1% FBS DMEM media with fungisome (PSF) was added to the plate. Media was changed every 3 days. AVM cells were obtained from Kai Jao of the University of Alabama, Birmingham.

siRNA Treatment

The 'siPORT *NeoFX* Transfection Agent' protocol from Applied Biosystems was used to treat both the AVM cells and the AV canals. siRNA's were diluted to 5uM stocks. For AV canals, the 24-well protocol was halved and followed resulting in a final volume of 250uL. Media 199 was used on explants. Explants were then incubated in suspension for 45 minutes at 37ºC. Explants were mixed by tapping every 15 minutes. After incubation with the siRNA the AV canals were seeded on collagen gels 'dry,' meaning complete media was removed from the gels prior and not added after the explants had attached. Explants were then incubated for 16 hours at 37ºC. At the end of the 16 hours, RNA was extracted using the TRIzol method. AV canals were treated with Snail2 and Runx2 siRNA at Stages 14 and 16.

AVM cells were treated using the protocol for 96-well plates, resulting in a final volume of 100uL. Cells were initially treated in suspension and allowed to attach overnight as micromasses. Every 3 days, siRNA was boosted by using the 6-well protocol, with a final volume of 2.5mL.

When boosting, cells were treated overnight and then complete DMEM media was added the next day. Runx2 siRNA was used on the micromasses. RNA was extracted using the E.Z.N.A. Total RNA Kit I.

RNA Extraction

RNA was extracted from AV canals using the Invitrogen TRIzol Plus RNA Purification Kit, (Cat No. 12183-555). RNA from AVM cells was extracted using the Omega Bio-Tek E.Z.N.A. Total RNA Kit I, (Product # R6834-02). After RNA extraction 1ug of RNA was used from samples to make cDNA for Real Time PCR experiments. cDNA was made using the qScript Reverse Transcriptase Reaction Mix from Quanta Biosciences, Inc. (Cat No. 95047-100). The concentration of cDNA was measured using the Invitrogen Quant-iT OliGreen ssDNA Assay Kit (Lot: 41320A).

Real Time PCR

Primers for RT-PCR were obtained from Integrated DNA Technologies, (IDT) and diluted to 1mM stocks. Working stocks were made at 50uM. 10ng of cDNA was used for each sample. FastStart SYBR Green Master from Roche Diagnostics (Lot: 12180420) was used in RT-PCR reactions.

Von Kossa Stain

Cells grown as micromasses were incubated with 1% silver nitrate solution, (1g silver nitrate to 100mL distilled water,) and placed under a UV light for 20 minutes. The cells were then rinsed several times with distilled water. Un-reacted silver was removed using 5% thiosulfate, (5g sodium thiosulfate to 100mL water,) for 5 minutes. Cells were then rinsed in distilled water once more. Calcium deposits were stained black or brown-black.

RESULTS

Runx2 and Epithelial to Mesenchymal Transition

Existing data on Runx2 focuses on its role as a transcription factor in the bone, and widely ignores its function in other tissues. We choose to focus on Runx2's possible function in the heart, after observing its expression in embryonic chicken hearts at the time of EMT (Figure 1). We also observed that of the two isoforms, which differ in only one exon at the 5' region, only Runx2 (I) is expressed in the heart (Figure 1, Andres Tavares.) Samples from the wing, whole embryo and heart were isolated and a Western blot analysis was done to look for the expression of each isoform. While both isoforms are expressed in the wing and the whole embryo, only Runx2 (I) is expressed in the heart (Figure 1, Andre Tavares). Immunofluorescent staining for Runx2 was performed to examine where the protein was expressed in the heart (Figure 1, Andre Tavares). We see expression of Runx2 in the endothelium, as well as the myocardium. After verifying that Runx2 was indeed expressed in the embryonic chicken heart, we tested whether or not it had a function during EMT.

In order to observe the EMT process AV canals were isolated from embryonic chicken hearts and grown on collagen gels after siRNA treatment. In explants treated with Runx2 siRNA we see more epithelial-like cells and less invaded cells than in the control siRNA treated explants (Figure 2, Andre Tavares). This observation suggested that Runx2 did have a role in EMT, as the process was slowed in the Runx2 siRNA treated group. To further explore Runx2 (I)'s function in EMT, we observed the effect of common signaling pathways on mRNA expression.

AV explants were treated with anti-TGFβ2, anti-TGFβ3, and noggin (which blocks BMP signaling), as well as TGFβ2, TGFβ3, and BMP-2 to see the effect on Runx2 mRNA expression (Figure 3, Andre Tavares). It was observed that anti-TGFβ2 caused a down-regulation of Runx2

(I) and the addition of TGFβ2 resulted in an up-regulation. This suggests a role for Runx2 (I) during the activation step of EMT, so expression levels of EMT- related transcription factors were observed to look for downstream targets of Runx2 (I).

We also showed the expression of different EMT markers with Runx2 siRNA treatment on Stage 14 explants, prior to EMT (Figure 4). EMT-related transcription factors, Snail1, Zeb1 and Twist1 were down-regulated in response to the treatment. Markers of mesenchymal cells, α-smooth muscle actin, collagen I and periostin also displayed decreased expression. VE-cadherin, an endothelial marker that is down-regulated with EMT was also reduced (Andre Tavares, unpublished data). Endothelial VEGF receptors Flk-1 and Flt-1 were also down-regulated and Flk-1, a receptor that is up-regulated during EMT in the heart was strongly decreased. Stage 14 explants are at an early stage of EMT and we had previously observed differences between this stage and somewhat older explants in a previous study of TGFβ receptor responses (Mercado-Pimental, et al., 2007). Accordingly, the expression patterns of these markers were also observed using Stage 16 AV canals.

When explants were treated with Runx2 siRNA at Stage 16 a down-regulation of Snail2, VE-cadherin, Flk-1 and ALDH1A1 was observed (Figure 5). Twist1 was the only marker up-regulated by the treatment. Because Snail2 has well-documented roles in EMT we also choose to observe how Snail2 siRNA affected Runx2 (I) and other EMT-related transcription factors in the embryonic heart.

Snail2 is a zinc-finger transcription factor which is up-regulated during EMT. When AV canals are treated with Snail2 siRNA at Stage 14, Runx2 (I), Zeb1 and Zeb2 decreased expression (Figure 6). Snail1 displayed an increase in expression. At Stage 16, Snail2 siRNA

explants displayed an increase in expression of Runx2 (I), Twist1 and Flk-1, and continued down-regulation of Zeb2. Taken all together, these data illustrate Runx2 (I)'s role during EMT in the embryonic chicken heart. To further explore the function of Runx2 in the heart, we also looked at the calcification process, as Runx2's known role involves bone formation and skeletal development.

Runx2 in Heart Valve Calcification

After showing that Runx2 (I) had a role during EMT, we decided to observe its effect on the calcification, while a focus on the functions of each isoform during the process. We first showed that when chick valves are isolated and grown to promote calcification, that Runx2 (II) is significantly up-regulated (Figure 7b, Reginald Doku unpublished data). To examine calcification in an easily manipulated culture system, we acquired mouse AVM cells (courtesy of K. Jao, Univ. of Ala., Birmingham). These cells were isolated from the cardiac cushions of an embryonic mouse containing a temperature-sensitive large T transgene. At 37ºC this immortalizing gene is inactive and the cells are a cardiac mesenchymal cell population, but at 33ºC the cells can be maintained as an immortal cell line. Micromass cultures were originally developed to explore calcification by chick limb mesenchymal cells (Ahrens, et al., 1977). We determined that AVM cells, when grown in micromass cultures at 10,000 cells/uL, also display calcification, as seen in the brown-yellow stains visualized by Von Kossa staining (Figure 7). L-cells are a mouse fibroblast cell line that did not calcify in micromass culture and therefore were used as a control. To better understand Runx2's role during calcification, we treated AVM cells with inhibitors for common developmental pathways.

AVM cells were again grown as micromasses and then treated with Runx2 siRNA (targeting both isoforms,) Runx2 (II) siRNA, noggin, anti-TGFβ, and DAPT (which blocks Notch

signaling) (Figure 8). Treatment with noggin and anti-TGFβ did not have an effect on the expression of Runx2 isoforms, Twist1, Cbfβ and Mgp. Twist1 expression was observed because of its roles during EMT and Mgp expression was measured as a calcification marker. Cbfβ is an important binding partner of Runx2, so its expression levels was also observed in order to see if it had any differential effect on the two isoforms. Both siRNA treatments resulted in the down-regulation of Runx2 (I), Runx2 (II), Twist1, and Cbfβ. In Runx2 siRNA treated cells Mgp remained unchanged, while Runx2 (II) siRNA decreased Mgp expression. DAPT was the only pathway inhibitor which down-regulated the expression of both Runx2 isoforms. Twist1 and Cbfβ were up-regulated in these samples and Mgp was relatively unchanged. After observing the effect of different inhibitors, we decided to observe how siRNA treatments affected calcification markers.

AVM cells were grown as micromasses and treated with Runx2 and Runx2 (II) siRNA at 5 and 12 days (Figure 9). As Runx2 (I) and Runx2 (II) differ by only a single exon, it was not possible to identify a siRNA that was specific for the Runx2 (I) isoform. The Runx2 siRNA would target both isoforms while the Runx2 (II) siRNA targeted the extra exon of the larger isoform. We found that treatment with Runx2 siRNA has a more profound effect on down-regulation of calcification markers, including periostin, osteonectin, osteopontin, and Mgp. Runx2 (II) siRNA was not very successful at decreasing expression, most likely as it was a less effective construct. Lastly, we decided to look at how noggin and anti-TGFβ treatment affected the calcification process at 5, 7 and 12 days.

While day 5 and 7 showed relatively no change in calcification markers, (much like what was observed in Figure 8,) some markers were down-regulated at 12 days. At 12 days, noggin treated cells showed an up-regulation of Runx2 (II) and Twist1, and a down-regulation of

periostin (Figure 10). Anti-TGFβ treatment caused the up-regulation of both isoforms, Twist1, and Mgp, and again a down-regulation of periostin. The data described above shows what signaling pathways may be important in regulating Runx2 isoforms during the calcification process in the heart.

DISCUSSION

While it is well-known that Runx2 has important roles during osteoblast differentiation in skeletal development, the roles of the individual isoforms are not well understood. Most of the literature on Runx2 also ignores potential isoform differences and their functions in non-osteogenic tissues of the body. This prompted us to explore Runx2's role in embryonic heart development, as well as the calcification of heart valve cells, with a focus on the specific functions of each isoform.

Our findings indicate that only the Runx2 (I) isoform is present in the embryonic heart at the time of EMT and is required for this process to occur, as seen in Runx2 siRNA treated AV canals. Observation that Runx2 (I) is significantly down-regulated when treated with anti-TGFβ2, but not as strongly by TGFβ3, suggests that Runx2 (I) expression is correlated with the activation step of EMT rather than the invasion step (Boyer, et al., 1999). When TGFβ3 is added to cultures we do see a smaller increase in Runx2 mRNA expression, but it is not as significant as when TGFβ2 is added. It is known that differences in potency of exogenous TGFβs can reflect isoform specificity *in vivo* but that all isoforms may produce a response *in vitro* (Roberts, et al., 1990).These data suggest that TGFβ2 is the principal mediator of Runx2 (I) expression *in vivo*.

After observing that treatment with Runx2 siRNA resulted in decreased invasion of endothelial cells into the collagen matrix, we decided to see whether inhibition of Runx2 affected

any transcription factors and other markers of EMT. AV canals were treated with both Runx2 and Snail2 siRNA at Stages 14 and 16 to see the effect on EMT markers during early and late phases of the EMT process. In Stage 14 explants, the inhibition of Runx2 resulted in the decreased expression of EMT-related transcription factors Twist1, Snail1, and Zeb1. Other transcription factors, such as Snail2 and Zeb2 were unaffected. The loss of these transcription factors, as well as the down-regulation of Runx2, also inhibited mRNA expression of VE-cadherin, collagen I, α-smooth muscle actin and periostin. Flk-1 and Flt-1, endothelial markers shown to be up-regulated and down-regulated, respectively, in response to EMT, were both down-regulated. (Stankunas, et al., 2010). The same markers were also measured for expression in Stage 16 explants treated with Runx2 siRNA.

In Stage 16 AV canals treated with Runx2 siRNA Snail2 was down-regulated and Twist1 was up-regulated. The rest of the transcription factors were largely unaffected. VE-cadherin, Flk-1 and ALDH1A1 were down-regulated in response to the treatment. The down-regulation of Snail2 supports its function as a positive regulator of Runx2 (Lambertini, et al., 2009). Twist1 may be up-regulated at this later stage when Runx2 function is no longer required. The down-regulation of VE-cadherin and Flk-1 suggest that the EMT process is still partially inhibited. ALDH1A1 was observed as a possible mesenchymal marker. Since most of the markers seem to be unaffected at a later stage of EMT, this data suggests that Runx2 has an early role in the activation step of EMT, and that the inhibition of Runx2 blocks EMT from occurring. Because Snail2 has familiar roles in EMT, we decided to also treat AV canals with Snail2 siRNA to see the effects on Runx2 (I) and the other transcription factors.

When treated with Snail2 siRNA at Stage 14, explants displayed decreased expression of Runx2 (I), Zeb1, and Zeb2, in concurrence with the inhibition of EMT. As it was previously

shown that Snail2 was a target of TGFβ (Romano and Runyan, 2000), this suggests that Snail2 may regulate Runx (I) but as Runx2 (I) regulated Snail2, there may be a feed-forward regulation between these two transcription factors. Surprisingly though, Snail1 displayed increased expression. This may suggest that its expression could be increased in order to compensate for the loss of the other transcription factors, but Snail1 has not been previously examined in EMT in the heart and its role is uncertain. Research has shown that Snail1 inhibits the expression of Runx2 during osteogenic differentiation (Park, et al., 2010). Therefore, the down-regulation of Runx2 (I) could be caused by the increased expression of Snail1, when Snail2 is blocked. The effect of Snail2 siRNA was also observed on Stage 16 AV canals.

At Stage 16, Zeb2 is the only transcription factor down-regulated in response to Snail2 siRNA. Runx2 (I), Twist1 and Flk-1 were all up-regulated in response to the treatment. An up-regulation of Runx2 (I) is somewhat unexpected, as Snail2 enhances the expression of Runx2 (I) by binding to the E-box sequences in both promoter regions (Lambertini, et al., 2009). Research has shown though, that Twist1, depending on the cell-type, can have either inhibitory, enhancing or no effect on Runx2 (Yuen, et al., 2008). The up-regulation of both Twist1 and Runx2 suggests that in endothelial tissue, Twist1 may positively regulate Runx2 (I) in order to promote EMT. Thus, while many of the EMT-associated transcription factors are found in the heart, the interactions between clearly need more examination.

While Flk-1 was up-regulated in these samples, Flt-1 was not down-regulated in agreement with their expression patterns for EMT (Stankunas, et al., 2010). This may mean that the ratio of these markers to one another is important to note, when determining if cells have undergone EMT. Further experiments are underway to explore the utility of Flk-1/Flt-1 ratios and expression of ALDH1A1 as markers of the transition to mesenchyme.

After observing that Runx2 (I) had a possible function in the activation step of EMT, we decided to observe its role in the calcification of heart valves, since Runx2's known role involves the regulation of bone formation. To explore Runx2's role in calcification of heart valves chick valves were cultured for seven days to promote calcification, (as described by Peacock, et al., 2010). It was observed that as calcification increased, so did the expression of Runx2 (II). Runx2 (I) also showed an expression increase, but to not the same extent as Runx2 (II). This prompted the exploration of the specific functions of each isoform further during the calcification process.

As an *in vitro* model, AVM cells were grown as micromasses to observe if their prolonged culture at a high cell density resulted in calcification. Von Kossa staining on AVM cells displayed some calcification at seven days, but greater levels of calcification are seen at twelve days. L-cells, (a mouse fibroblast cell line which doesn't adhere to one another,) were used as a control and displayed no calcification. This experiment confirmed our use of AVM cells to determine Runx2 isoform's role in the calcification of heart valves.

We started by trying to determine which pathways, with known roles in bone formation, could be preferentially regulating the two isoforms. This was done by blocking common signaling mechanisms with antibodies and inhibitory protein treatments. Expression levels of Runx2 (I), Runx2 (II), Twist1, Cbfβ and Mgp were observed. Twist1 was observed because of its role during osteoblast differentiation, while Mgp was used as a marker for calcification (Li, et al., 2008, Yuen, et al., 2009). Cbfβ was examined because of its role as an important binding partner of Runx2 (Kanatani, et al., 2006).

While TGFβ2 seemed to be important during Runx2's role in the EMT process, the TGFβ pathways did not have an effect on Runx2 isoforms during calcification at day 7. Noggin treatment, which blocks BMP signaling, also did not have an effect on the Runx2 isoforms and

only slightly decreased the other markers. DAPT, which blocks Notch signaling, had the most effect on down-regulating the Runx2 isoforms. It also showed more of an effect on decreasing Runx2 (I) than Runx2 (II). This may hint towards a possible mechanism by which Notch signaling could regulate each isoform differently. For instance, Notch could be a negative regulator of Runx2 (II) and a positive regulator of Runx2 (I). The DAPT treatment also increased the expression of Twist1 and Cbfβ. This up-regulation may be a result of signaling from the TGFβ and BMP pathways, as both are known to induce Twist1 expression.

Runx2 (II) siRNA treatment resulted in the down-regulation of both isoforms, Twist1 and Cbfβ and Mgp, while the siRNA which blocks both isoforms knocked down all the genes, except Mgp. This suggests that targeted inhibition of Runx2 (II) better targets the down-regulation of Mgp, a calcification marker. Cbfβ, which binds to the Runx2 Runt domain, was down-regulated by both siRNA treatments in accordance with its function as a binding partner. Twist1 is down-regulated in both cases, which is surprising since in some osteoblast cell lines it blocks Runx2 expression (Yuen, et al., 2008). This again suggests that in the heart, Twist1 is a positive regulator of Runx2 and requires its expression. To further explore the Runx2 isoform role in calcification, AVM cells in micromass were treated with Runx2 and Runx2 (II) siRNA and then probed for calcification markers.

AVM cells were again grown as micromasses and probed for calcification markers at days five and twelve. Day five was chosen as the Von Kossa stain exhibited no calcification of the cells, while day twelve demonstrated the most calcification. It also appears that treatment with Runx2 siRNA (which blocks both isoforms) better blocks the calcification process at days five and twelve. This suggests that Runx2 (II) is not solely responsible for causing heart valve calcification; Runx2 (I) is partly responsible for the process. siRNA treatment effects Runx2 (I)

more, suggesting Runx2 (II) may be up-regulated in response to calcification. Runx2 (II) siRNA at five and twelve days does not block calcification markers, and also does not seem to block Runx2 (II) expression very well. In the future, new siRNA for Runx2 (II) will be made and tested for more specific and increased knockdown.

To better understand isoform signaling, we also treated AVM cell micromass cultures with noggin and pan-antibody (which blocks TGFβ signaling) for 5, 7 and 12 days. While treatments cause markers to remain relatively unchanged at days 5 and 7, at day 12 Runx2 isoforms, Twist1, and Mgp all displayed an up-regulation. Periostin was down-regulated with both treatments, suggesting that the calcification process was being blocked. This data also shows that in the absence of BMP (noggin) and TGFβ (pan-ab) signaling both isoforms are up-regulated, showing that the regulation of Runx2 during the calcification process in the heart by 12 days is somewhat dependent on these two mechanisms. This suggests that early in the calcification process, Notch signaling is more important in regulating the two isoforms, while later BMP signaling, in conjunction with TGFβ signaling may be controlling isoform expression. This data does show though, that inhibition of the BMP pathway increases Runx2 (II) expression more than Runx2 (I) expression, meaning that this pathway could be important in the specification of which isoform is expressed. To better understand this process, cultures will also be treated with DAPT at 12 days. Twist1 expression is again up-regulated in accordance with the hypothesis that it is a positive regulator of Runx2 in the heart.

Through investigating Runx2's role in embryonic heart development and heart valve calcification novel functions for the transcription factor were determined. Runx2 (I) was shown to be regulated by TGFβ2 in the embryonic heart and also to have a role in the activation step of EMT during development. In regards to calcification, the up-regulation of Runx2 (II) in the

heart suggests an abnormal regulation which may involve the Notch signaling pathway, as well as BMP and TGFβ signaling. Further work will be focused on better elucidating the specific targets of Runx2 isoforms in each of these processes.

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FIGURE LEGENDS

Fig. 1. Runx2 (I) is expressed in the chick embryo wing, whole embryo and heart and is also present in the AVM region at the time of EMT. a) Runx2 (I) is present in the embryo wing (A), whole embryo (B), and heart(C), while Runx2 (II) is only present in the wing and embryo. b) Runx2 isoforms differ in their alternative splice sites and the presence of a single exon at the 5' end. c) cj-cardiac jelly, e-endothelium, m-myocardium, l-lumen. Red: Runx2 antibody (Santa Cruz). Blue: nuclear stain (DAPI). *Figure courtesy of Andres Tavares.*

Fig. 2. Runx2 siRNA treatment blocks EMT and cell separation by endothelia. a) Shows the surface of the collagen gel culture with endothelial activation while b) shows invaded mesenchymal cells below the gel surface. In c) the endothelium remains polygonal and few, if any cells, have invaded the matrix in d). The graph to the right shows that Runx2 siRNA treated cells display decreased invasion into the collagen gel matrix. *Figure courtesy of Andres Tavares.*

Fig. 3. Runx2 (I) expression is regulated by TGFβ2 in avian heart explants. a) Stage 14 chick heart explants were grown on collagen gel cultures and treated for 4 hours. Runx2 (I) expression was inhibited by anti-TGFβ2 treatment, and b) elevated in TGFβ2 and TGFβ3 treated cultures. *Figure courtesy of Andres Tavares.*

Fig.4. Inhibition of Runx2 by siRNA treatment reduces expression of EMT-related transcription factors and EMT markers. a) Runx2 siRNA reduces expression of EMT- related transcription factors, Snail1, Zeb1 and Twist1. b) Reduction in Runx2 (I) expression decreases the expression of EMT markers such as, VE-cadherin, α-SMA, collagen I, periostin, Flk-1 and Flt-1.

Fig. 5. At Stage 16, Runx2 siRNA treated AV canals display a knockdown of Snail2, VE-cadherin, Flk-1 and ALDH1A1, while Twist1 was up-regulated. a) AV canals were isolated at Stage 16 and treated with Runx2 siRNA. Of the EMT-related transcription factors only Snail2 was down-regulated and Twist1 was up-regulated. b) EMT markers VE-cadherin, Flk-1 and ALDH1A1 were down-regulated in response to treatment.

Fig. 6. AV canals treated with Snail2 siRNA at stage 14 display decreased expression of some EMT-related transcription factors, while treatment at stage 16 shows somewhat of an opposite effect. a) AV canals treated with Snail2 siRNA at stage 14 display decreased expression of EMT-related transcription factors Zeb1 and Zeb2, but increased expression of Snail1. Runx2 (I) also shows decreased expression. b) At Stage 16, Runx2 (I) shows about a 60% increase, while Zeb2 remains down and Twist1 increases. EMT marker Flk-1 also increases.

Fig. 7. Valve tissues display increased Runx2 (II) expression. a) AVM cells were cultured as micromasses for 12 days and then von Kossa stained to observe calcification (arrows). L-cells were used as a control. Calcification was accompanied by expression of Runx2 (II) (not shown). b) AV valves were isolated from E6 chick hearts and grown in explant culture. Valves displayed calcification (not shown). This coincided with an up-regulation of Runx2 (II) at Day 5. Runx2 (II) increases expression by Day 7. *Figure 7b courtesy of Reginald Doku.*

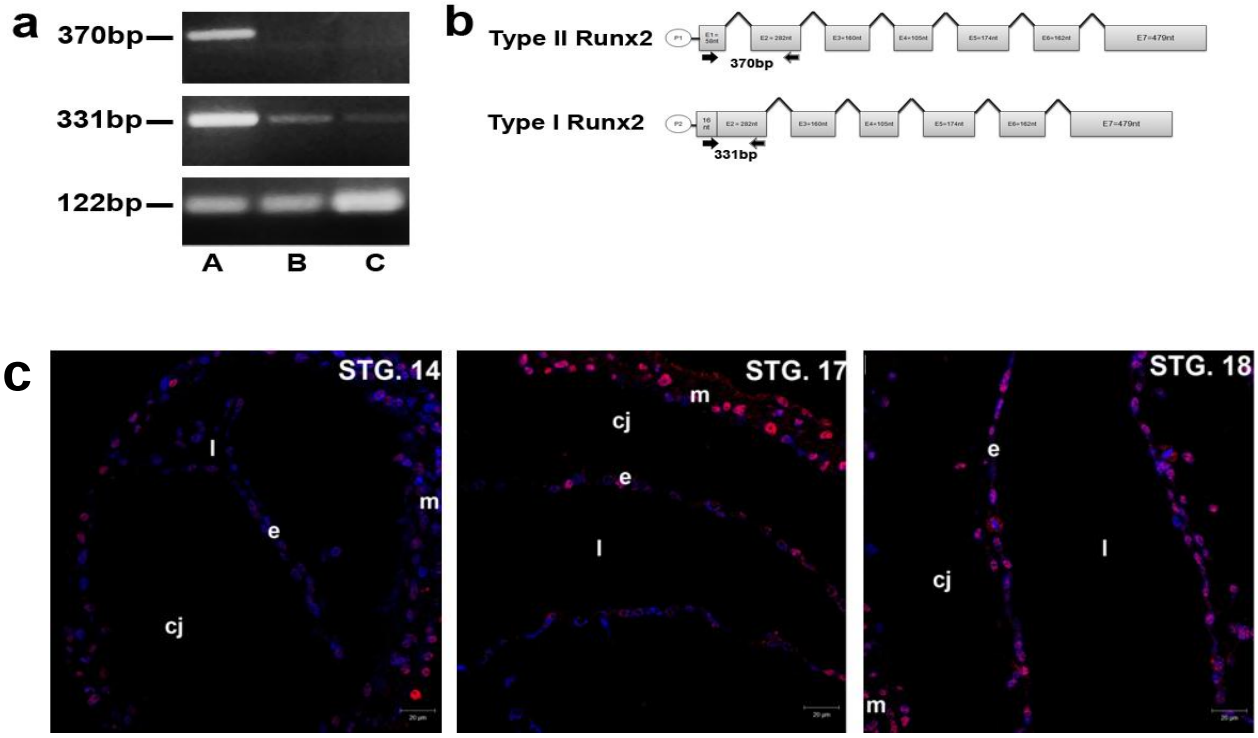
Fig.8. Runx2 isoform expression is down-regulated by Runx2 siRNA's and DAPT treatment. a) Treatment with Runx2 siRNA (blocks both isoforms) on Day 7 micromass cultures decreases the expression of Runx2 (I), Runx2 (II), Twist1 and Cbfβ, while Mgp remains unchanged. b) Runx2

(II) siRNA down-regulates all markers. c) Noggin (which blocks the BMP pathway) causes the down-regulation of Twist1, Cbfβ, and Mgp, while Runx2 (I) and Runx2 (II) remain relatively unchanged. d) Anti-TGFβ treatment did not affect the isoforms, but caused a slight down-regulation in Twist1 and a slight increase in expression of Cbfβ and Mgp. e) DAPT (which inhibits Notch signaling) decreased expression of Runx2 (I), while Runx2 (II) remained unchanged, along with Mgp. Twist1 and Cbfβ increased expression.

Fig. 9 Knockdown of Runx2 by siRNA down-regulates calcification markers in micromass cultures. a) AVM cells were again grown as micromasses and treated with Runx2 (II) and Runx2 siRNA at day 5 and 12. Runx2 siRNA which targets both isoforms is better at blocking the calcification of AVM cells grown in micromass. The more down-regulated the isoforms, the more down-regulated calcification markers, such as periostin, osteonectin, osteopontin, and MGP, are.

Fig. 10. Treatment with noggin and anti-TGFβ result in an up-regulation of Runx2 isoforms. a), b), c) AVM cells were grown as micromasses and treated with noggin and pan-ab (anti-TGFβ) for 5, 7, and 12 days. a), b) At days 5 and 7 markers remained relatively unchanged except periostin (POSTN) which was up-regulated at day 7. c) At day 12 both noggin and pan-ab treatments resulted in an up-regulation of Runx2 (I), Runx2 (II), Twist1, and Mgp, and a down-regulation of POSTN. Noggin treatment had a greater effect on Twist1 and POSTN, while the pan-ab treatment had a more pronounced effect on the Runx2 isoforms and Mgp. Cbfβ remained unchanged with both treatments at day 12.

Figure 1.



Western and immunofluorescence courtesy of Andre Tavares.

Figure 2.

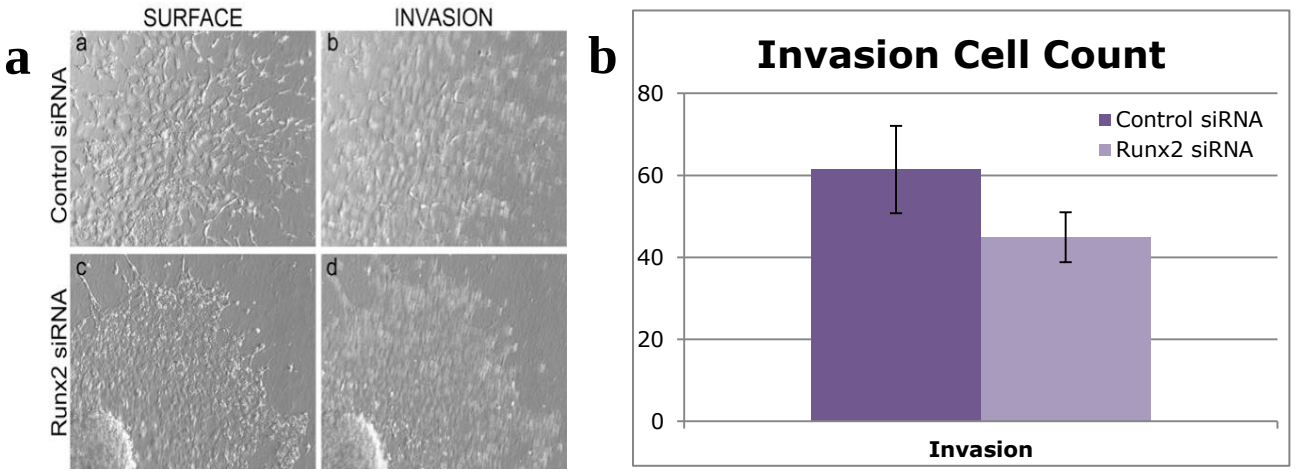


Figure courtesy of Andre Tavares.

Figure 3.

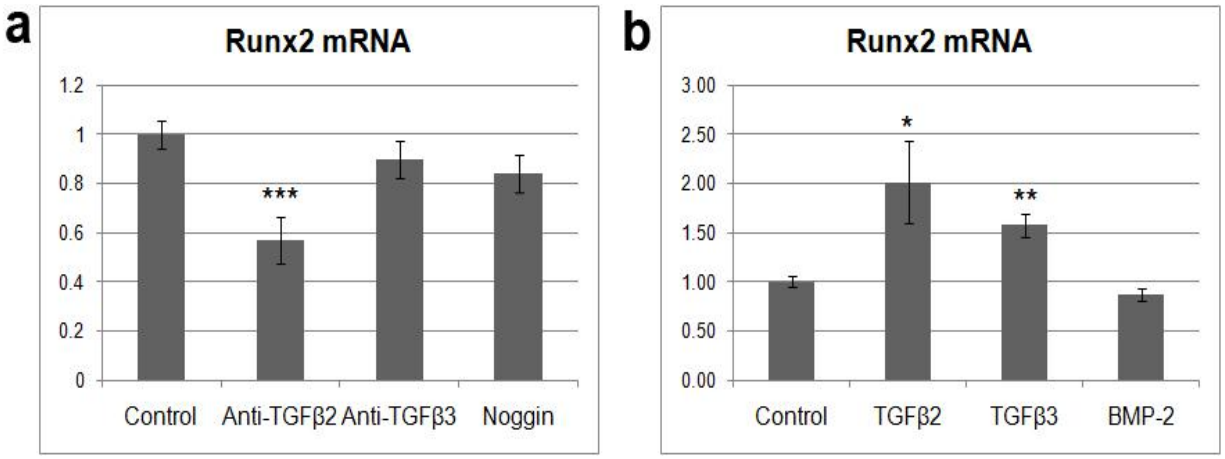
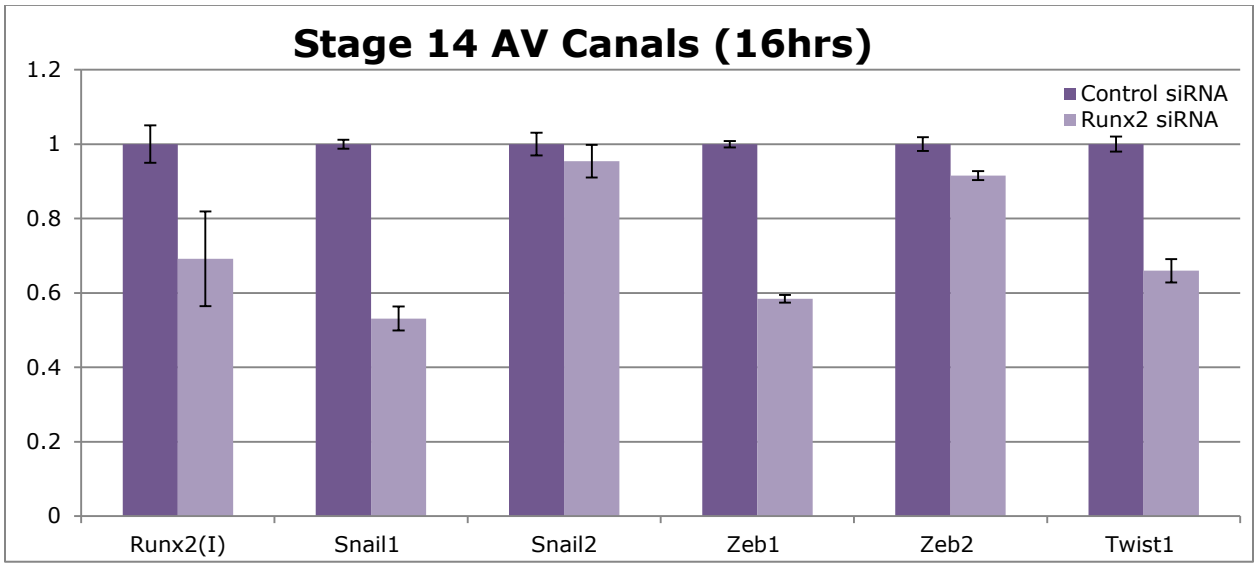


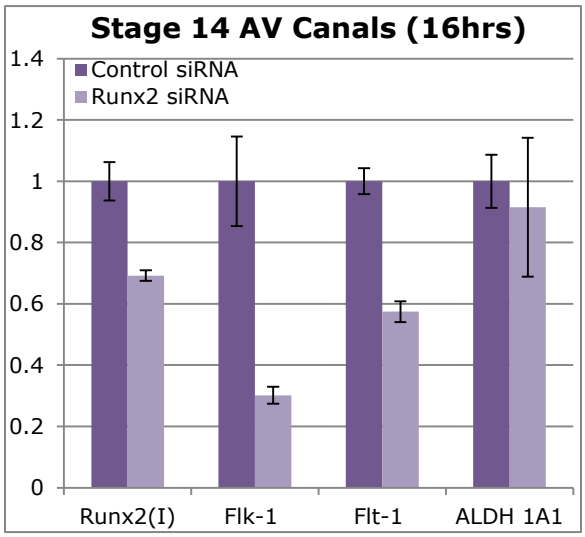
Figure courtesy of Andres Tavares.

Figure 4.

a


Stage 14 AV Canals (16hrs)
Control siRNA
Runx2 siRNA
1.2
1
0.8
0.6
0.4
0.2
0
Runx2(I)
Snail1
Snail2
Zeb1
Zeb2
Twist1

b


Stage 14 AV Canals (16hrs)
Control siRNA
Runx2 siRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Runx2(I)
Flk-1
Flt-1
ALDH 1A1

c

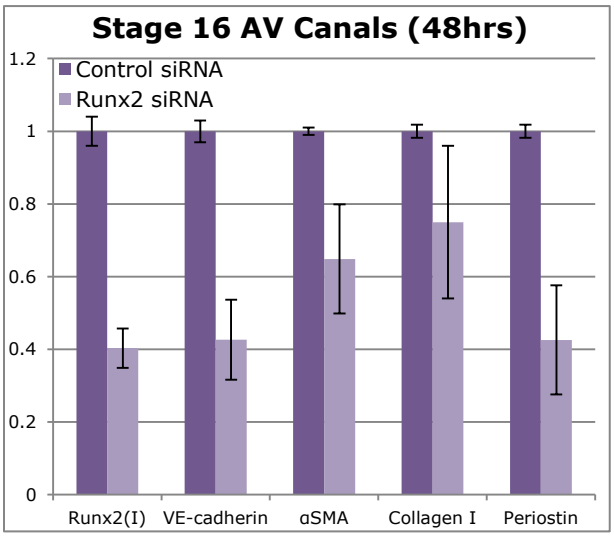
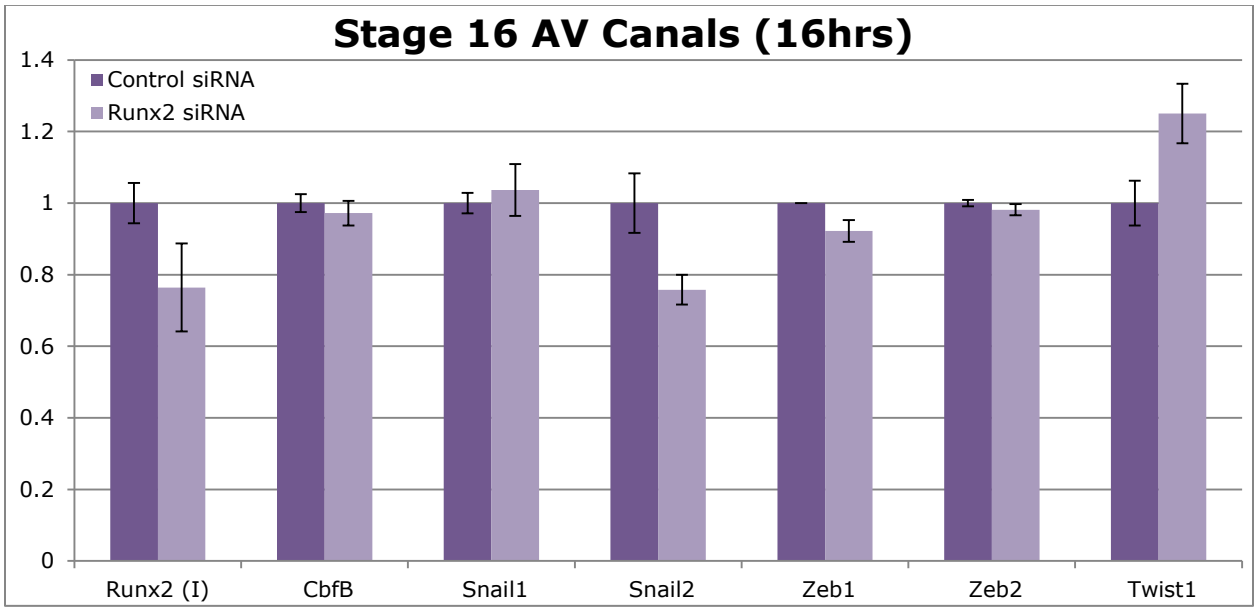

Stage 16 AV Canals (48hrs)
Control siRNA
Runx2 siRNA
1.2
1
0.8
0.6
0.4
0.2
0
Runx2(I)
VE-cadherin
αSMA
Collagen I
Periostin

Figure 5.

a


Stage 16 AV Canals (16hrs)
Control siRNA
Runx2 siRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Runx2 (I)
CbfB
Snail1
Snail2
Zeb1
Zeb2
Twist1

b

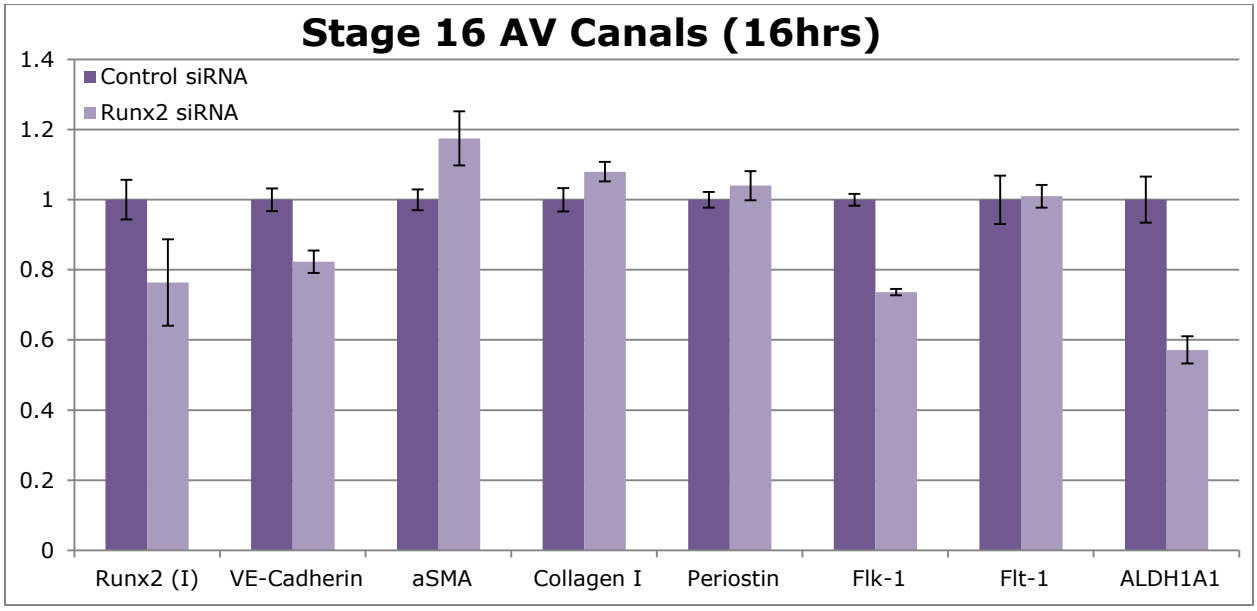
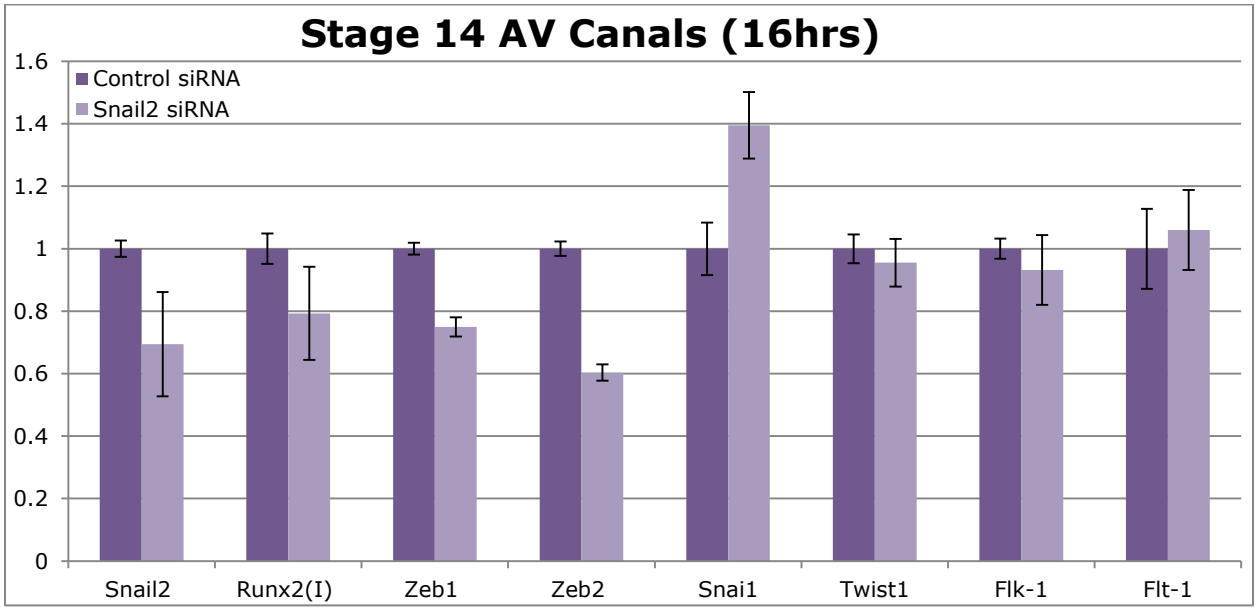

Stage 16 AV Canals (16hrs)
Control siRNA
Runx2 siRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Runx2 (I)
VE-Cadherin
aSMA
Collagen I
Periostin
Flk-1
Flt-1
ALDH1A1

Figure 6.

a


Stage 14 AV Canals (16hrs)
Control siRNA
Snail2 siRNA
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Snail2
Runx2(I)
Zeb1
Zeb2
Snai1
Twist1
Flk-1
Flt-1

b

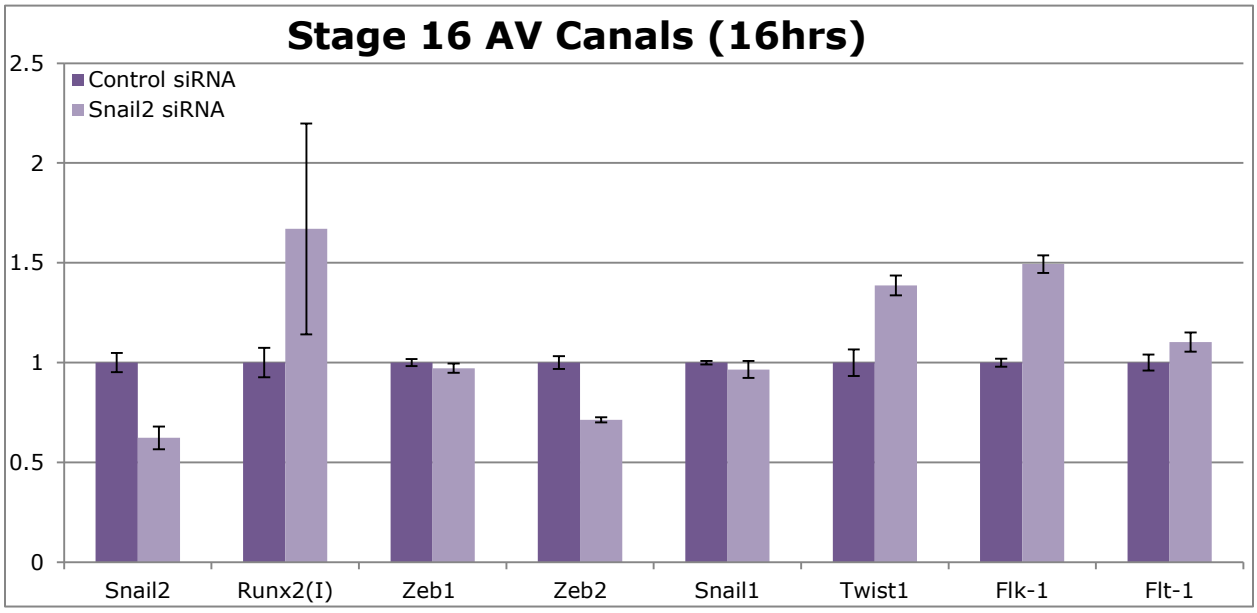
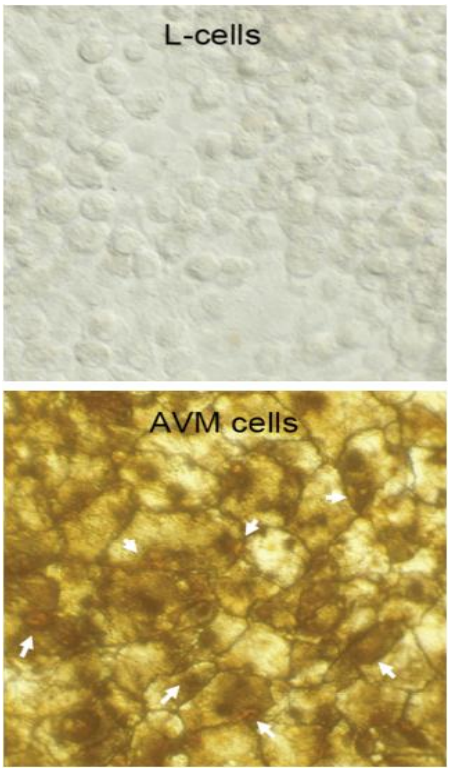

Stage 16 AV Canals (16hrs)
Control siRNA
Snail2 siRNA
2.5
2
1.5
1
0.5
0
Snail2
Runx2(I)
Zeb1
Zeb2
Snail1
Twist1
Flk-1
Flt-1

Figure 7.

a



b

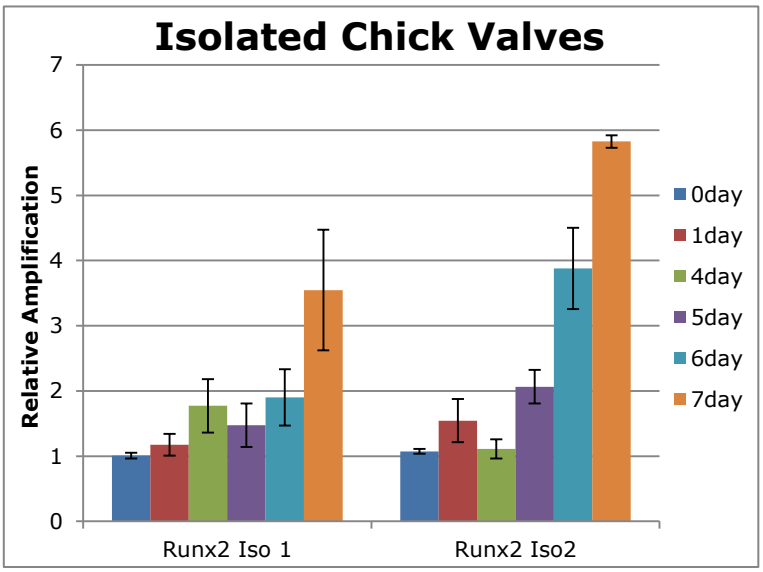


Figure 7b courtesy of Reginald Doku.

Figure 8.

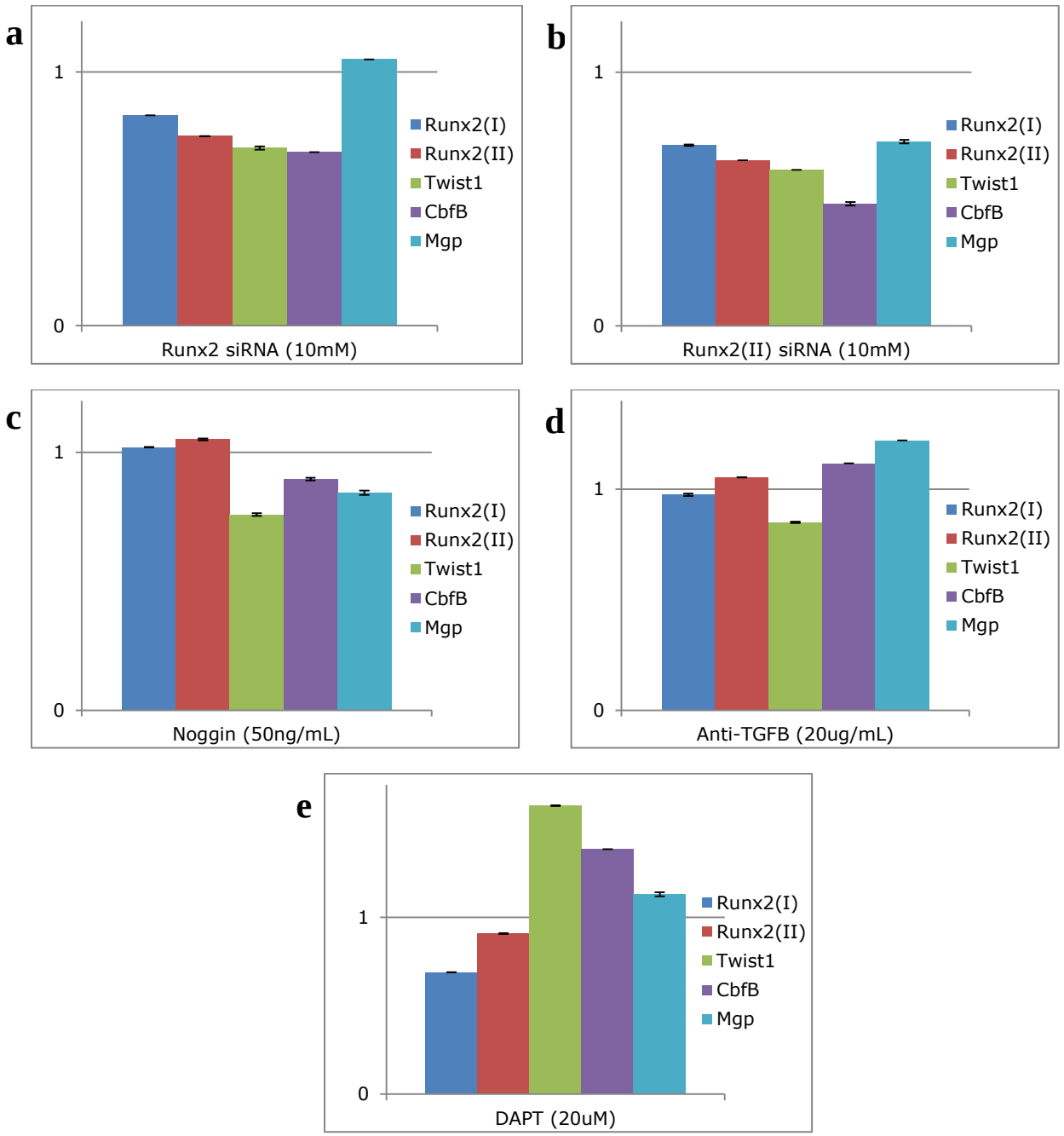

a
1
0
Runx2 siRNA (10mM)
Runx2(I)
Runx2(II)
Twist1
CbfB
Mgp
b
1
0
Runx2(II) siRNA (10mM)
Runx2(I)
Runx2(II)
Twist1
CbfB
Mgp
c
1
0
Noggin (50ng/mL)
Runx2(I)
Runx2(II)
Twist1
CbfB
Mgp
d
1
0
Anti-TGFB (20ug/mL)
Runx2(I)
Runx2(II)
Twist1
CbfB
Mgp
e
1
0
DAPT (20uM)
Runx2(I)
Runx2(II)
Twist1
CbfB
Mgp

Figure 9.

a

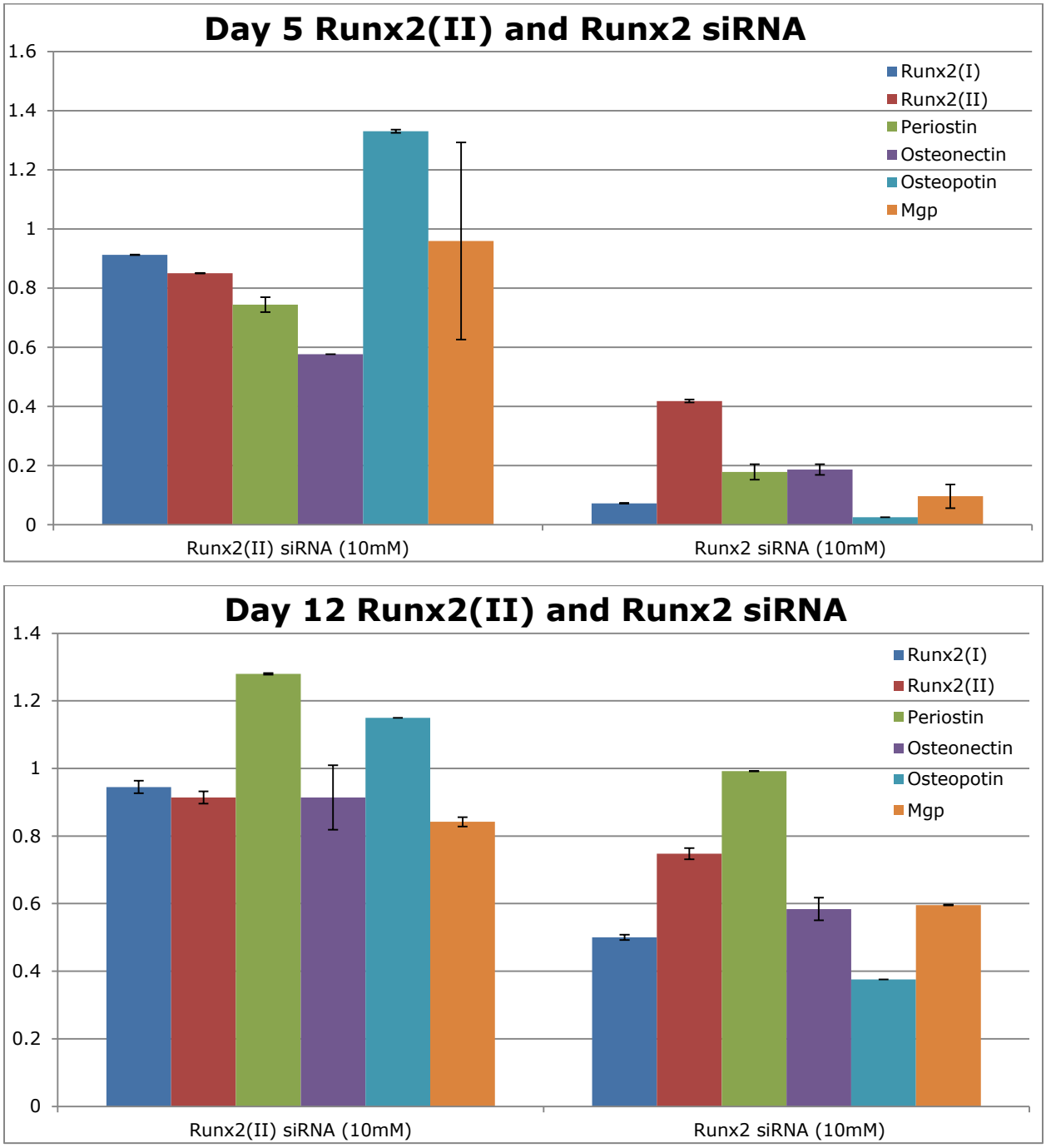
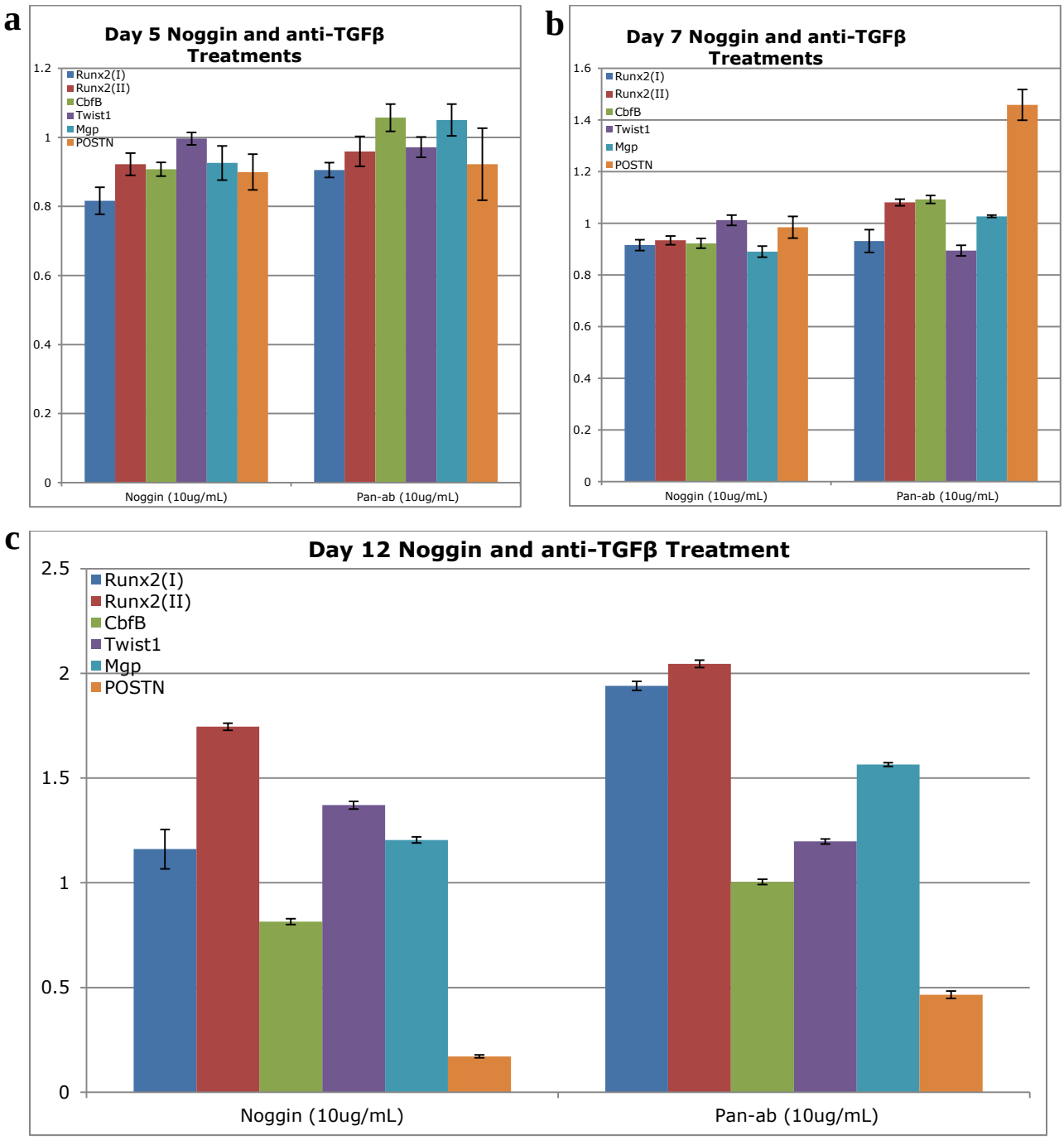

Day 5 Runx2(II) and Runx2 siRNA
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Runx2(I)
Runx2(II)
Periostin
Osteonectin
Osteopotin
Mgp
Runx2(II) siRNA (10mM)
Runx2 siRNA (10mM)
Day 12 Runx2(II) and Runx2 siRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Runx2(I)
Runx2(II)
Periostin
Osteonectin
Osteopotin
Mgp
Runx2(II) siRNA (10mM)
Runx2 siRNA (10mM)

Figure 10.


a
Day 5 Noggin and anti-TGFβ Treatments
Runx2(I)
Runx2(II)
CbfB
Twist1
Mgp
POSTN
1.2
1
0.8
0.6
0.4
0.2
0
Noggin (10ug/mL)
Pan-ab (10ug/mL)
b
Day 7 Noggin and anti-TGFβ Treatments
Runx2(I)
Runx2(II)
CbfB
Twist1
Mgp
POSTN
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Noggin (10ug/mL)
Pan-ab (10ug/mL)
c
Day 12 Noggin and anti-TGFβ Treatment
Runx2(I)
Runx2(II)
CbfB
Twist1
Mgp
POSTN
2.5
2
1.5
1
0.5
0
Noggin (10ug/mL)
Pan-ab (10ug/mL)