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RUNX2-I is an early regulator of epithelial-mesenchymal cell transition in the chick embryo

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Abstract

Background—Though normally linked to bone and cartilage development, the Runt-related transcription factor, RUNX2, was reported in the mouse heart during development of the valves. We examined RUNX2 expression and function in the developing avian heart as it related to the epithelial-mesenchymal transition (EMT) in the atrioventricular canal. EMT can be separated into an activation stage involving hypertrophy and cell separation and an invasion stage where cells invade the extracellular matrix. The localization and activity of RUNX2 was explored in relation to these steps in the heart. As RUNX2 was also reported in cancer tissues, we examined its expression in the progression of esophageal cancer in staged tissues.

Results—A specific isoform, RUNX2-I, is present and required for EMT by endothelia of the atrioventricular canal. Knockdown of RUNX2-I inhibits the cell-cell separation that is characteristic of initial activation of EMT. Loss of RUNX2-I altered expression of EMT markers to a greater extent during activation than during subsequent cell invasion. TGF β 2 mediates activation during cardiac endothelial EMT. Consistent with a role in activation, RUNX2-I is regulated by TGF β 2 and its activity is independent of similarly expressed Snai2 in regulation of EMT. Examination of RUNX2 expression in esophageal cancer showed its upregulation concomitant with the development of dysplasia and continued expression in adenocarcinoma.

Conclusions—This data introduces the RUNX2-I isoform as a critical early transcription factor mediating EMT in the developing heart after induction by TGF β . Its expression in tumor tissue suggests a similar role for RUNX2 in the EMT of metastasis.

Keywords

EMT; RUNX2; heart; AVC

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INTRODUCTION

Epithelial-mesenchymal cell transition (EMT) is a regulated cellular process wherein epithelial cells lose cell-cell adhesions, change shape to a stellate or fibroblastic morphology and become invasive (Thiery et al., 2009; Nieto, 2011). In the embryo, EMT provides a mechanism utilized for gastrulation, neural crest cell formation and in other areas of the embryo to produce three-dimensional structures (Hay and Zuk, 1995). EMT reoccurs in the adult as a component of the pathologies of metastasis and fibrosis (Kalluri and Neilson, 2003; Thiery et al., 2009). EMT in the embryonic heart is a prototypical example of this process (Yang and Weinberg, 2008). Cardiac endothelial cells lining the atrioventricular canal (AVC) undergo an EMT, invade the underlying matrix and form valve progenitors (Markwald et al., 1984). This EMT is mediated by TGF β superfamily members, including TGF β , TGF β 3 (Potts and Runyan, 1989; Potts et al., 1991; Boyer et al., 1999a), and BMP-2 (Yamagishi et al., 1999), and involves many of the molecules and signal transduction mechanisms common to other EMTs (Person et al., 2005; Nieto, 2011). Studies in the Runyan laboratory utilize an in vitro analysis with collagen gels to explore mechanisms involved in transition from endothelial cells to cardiac mesenchyme (Person et al., 2005; Mercado-Pimentel and Runyan, 2007; Lencinas et al., 2011).

Runt-related transcription factors (RUNX) are a family of regulators that include RUNX1, RUNX2 and RUNX3. All members contain a conserved DNA binding domain, the Runt domain (Ito et al., 2015). RUNX2, also called OSF2 or CBF α 1, is expressed in mineralizing tissues, being essential for osteoblast maturation (Komori et al., 1997). This transcription factor is also expressed in other embryonic tissues as well the developing vasculature (Zhao et al., 2002; Bronckers et al., 2005; Jeong et al., 2008). RUNX2 is over-expressed in different types of cancers, including breast cancer and prostate cancer cell lines that metastasize to bone (Pratap et al., 2005; Baniwal et al., 2010; Mendoza-Villanueva et al., 2011). RUNX2 also appears to induce oncogenic transformation of human bone marrow endothelial cells (Vitolo et al., 2007) and of thyroid epithelia (Niu et al., 2012). RUNX2 is also up-regulated in calcified aortic valves (Garg, 2006; Yang et al., 2009). The wide expression of RUNX2 in calcified tissues has led to an emphasis on osteoblastic differentiation and little is known of other roles. There are two RUNX2 isoforms that differ by transcription start site: isoform I (RUNX2-I), expressed in osteoblastic, pre-osteoblastic and non-osteoblastic mesenchymal cells and isoform II (RUNX2-II), a largely bone-specific isoform (Li and Xiao, 2007; Zhang et al., 2009; Okura et al., 2014). Alternative splicing is also responsible for additional and poorly understood isoforms (Terry et al., 2004). Of interest in a TGF β -mediated cellular process, RUNX2 interacts with SMADs to regulate gene expression (Ito and Miyazono, 2003). We were intrigued by the localization of *RUNX2* in the embryonic heart and set out to investigate its function during EMT (Gitler et al., 2003). An initial screen, using siRNA, suggested functional role for RUNX2 during EMT.

In the present study, we explore expression and function of RUNX2 during heart valve development in the chick embryo. RUNX2-I is expressed by the endothelial cells lining the AVC and the mesenchymal cells invading the cardiac jelly during EMT. This transcription factor is TGF β 2-regulated. Inhibition of RUNX2 mRNA expression disrupts EMT at an early stage before loss of cell-cell adhesion. Examination of marker gene expression

suggests that RUNX2 regulation can be distinguished from Snai2 regulation of EMT and that both gene transcripts are involved in early stages of EMT. To explore the relevance of the observation to metastatic mechanisms, we examined staged human esophageal tissues in the transition from normal epithelium to metastatic esophageal adenocarcinoma, a carcinoma reported to involve TGF β signaling (Onwuegbusi et al., 2006; von Rahden et al., 2006). RUNX2-I expression appears coincident with the transition from columnar epithelia (Barrett's Esophagus) to high-grade dysplasia. This distribution is consistent with a conserved role in an early stage of EMT in both development and pathology. These data suggest that RUNX2 expression in human tumors is related to the process of EMT in metastasis rather than targeting to bone tissue.

RESULTS

RUNX2 is expressed in the AVC region of the developing heart

For a spatiotemporal localization of RUNX2, Hamburger and Hamilton stage 14, 17, 18 and 20 chicken embryo hearts were dissected and processed for immunofluorescent staining. This interval covers the period between initiation of tissue interaction leading to EMT and the population of the cardiac cushions (valvular primordia) (Markwald et al., 1984). A monoclonal antibody against RUNX2 (51.7 kDa) was used in this assay. The antibody detected a band of approximately 50kDa by western blot using total protein of stage 17 chick embryo tissue (data not shown). RUNX2 is required for proper chondrogenesis and osteogenesis (Komori et al., 1997; Stricker et al., 2002) and hair follicle development in mice (Glotzer et al., 2008). As a positive control, tissues of E13 chick embryo wings were immunostained with the antibody. A nuclear protein was marked in osteoblasts in the region of endochondral ossification (arrows in Fig. 1G) and in cells in the feather papilla, homologous to hair follicles in chicken (arrowheads in Fig. 1H), confirming the antibody is recognizing chicken RUNX2.

In the AVC region of the chick heart, RUNX2 was detected in the myocardium at all stages (Fig. 1A-D). In endothelial cells, low expression of RUNX2 is detected by stage 14 (Fig 1A). This expression increases by stage 17 (Fig. 1B) and stage 18 (Fig. 1C), persisting until stage 20 (Fig. 1D). Mesenchymal cells that had delaminated from the endocardium and that were migrating within the underlying extracellular matrix, or cardiac jelly, were also found to express this transcription factor (Fig. 1C and D). In the OFT region of the chick heart, RUNX2 expression is also detected in endothelial and mesenchymal cells (Fig. 1E).

RUNX2 was also localized in activated endothelial cells (Fig. 1F) and mesenchymal cells (data not shown) derived from AVC explants cultured on a collagen gel lattice. Cells were counterstained with DAPI (nuclei) and phalloidin (F-actin) for cell morphology. RUNX2 co-localizes with DAPI in both cell types (Fig. 1F and data not shown). Although low RUNX2 signal was detected in the cell cytoplasm, the antibody detected RUNX2 mainly in the nuclei of these cells. Taken together, the results argue that RUNX2 is present during the regulation of AVC EMT in endothelial and mesenchymal cells and that changes in endothelial cell expression are concomitant with EMT.

RUNX2-I is the isoform expressed in the chick heart

To further test if *RUNX2* is present in the chicken embryo heart at the time of EMT, whole-mount in situ hybridization was performed using an RNA probe specific for chicken *RUNX2* mRNA. Figure 2A shows that *RUNX2* is expressed in different regions of a stage 18 chicken embryo, including the limb buds (arrowhead) and heart (arrows).

RUNX2 is commonly known to be an osteogenic factor. Type II *RUNX2* (*RUNX2-II*) is the bone-specific isoform while type I *RUNX2* (*RUNX2-I*) is broadly expressed (Li and Xiao, 2007). To verify which isoform is expressed during heart development in the chick embryo, primers specific for each isoform were designed (Fig. 2B). Since only *RUNX2-II* is sequenced for the chicken (NM_204128), the human and mouse *RUNX2-I* sequences were used as reference for primer design as well. RT-PCR was performed with tissue from E13 chick wing (Fig. 2C-a), stage 17 chick embryo (Fig. 2C-b) and stage 17 chick hearts (Fig. 2C-c). As expected, at E13, chick wing cells are expressing both isoforms (Fig. 2C-a). At stage 17, *RUNX2-I* is expressed in the chick embryo and heart (Fig. 2C-b, c).

RUNX2-I is TGF β -regulated in vitro

That *RUNX2* is expressed by endothelial and mesenchymal cells during the TGF β -regulated EMT (Potts and Runyan, 1989; Boyer et al., 1999a; Ma et al., 2005) suggests *RUNX2* is regulated by TGF β -family members. To examine whether *RUNX2* is involved in TGF β signaling, cultured AVC explants were co-stained for *RUNX2* and pSmad1/5/8 (BMP signaling mediators, Fig. 3A-C) or pSmad2/3 (TGF β signaling mediators, Fig. 3D-F). Activated endothelial cells on the gel surface showed co-expression of *RUNX2* and pSmad1/5/8 (Fig. 3C) and *RUNX2* and pSmad2/3 (Fig. 3F) in the cell nuclei showing cells that express *RUNX2* are receiving TGF β and/or BMP signal.

To test whether TGF β signaling regulates *RUNX2* expression, we examined the effect of exogenous TGF β 2, TGF β 3 or BMP-2 on *RUNX2* mRNA expression. Culture media with TGF β 2, TGF β 3 or BMP-2 were added to AVC explant cultures and the level of *RUNX2* mRNA was assessed by quantitative real-time PCR (qPCR) (Fig. 3G). TGF β 2 addition caused a significant ~2-fold increase in *RUNX2* mRNA ($p < 0.05$). Addition of TGF β 3 also caused a significant ~1.5-fold increase in *RUNX2* message ($p < 0.01$). BMP-2 did not cause significant changes in *RUNX2* mRNA ($p=0.116$). Next, we used neutralizing antibodies against TGF β 2 and TGF β 3 to block these ligands. To inhibit BMP signaling, cultures were treated with Noggin. The level of *RUNX2* mRNA present was compared with control cultures by qPCR (Fig. 3H). Only anti-TGF β 2 caused a significant effect on *RUNX2* message (~40% decrease of *RUNX2* mRNA, $p < 0.001$; anti-TGF β 1, $p=0.273$; Noggin, $p=0.837$). Taken together, the data shows that *RUNX2* is induced by TGF β 2 and suggests that this factor is part of the chick AVC EMT activation step in vitro.

RUNX2-I inhibition disrupts EMT in vitro

That *RUNX2* is present in endothelial and mesenchymal cells during the AVC EMT suggests this molecule is required for proper EMT initiation. Therefore, we decided to assay the function of this transcription factor using specific siRNAs. Stage 14 chicken AVC explants were transfected with 3 different siRNAs against *RUNX2* and placed on a collagen

gel lattice. Control explants were transfected with a scrambled siRNA. The level of *RUNX2* mRNA inhibition varied from 40% to 80% between experiments (~60% decrease in *RUNX2* message, $p < 0.001$, Fig. 4H).

RUNX2 siRNA-transfected explants showed inhibition of EMT when analyzed at 24h (Fig. 4A-F) and 48h after explants were placed on gels (data not shown). In control explants an average of 156.71 activated endothelial cells and mesenchymal cells were counted on the gel surface (S.E.M.=18.83; arrowheads in Fig. 4A, C, G) and inside the gel (arrows in Fig. 4E, G). In *RUNX2*-siRNA treated explants, on the gel surface, fewer activated endothelial cells (arrowheads in Fig. 4B, D, G) and mesenchymal cells (arrows in Fig. 4F, G) were observed (average=77.00 cells; S.E.M.=9.07; ~51% decrease; $p < 0.01$). *RUNX2*-siRNA treatment had a greater effect in the number of activated endothelial cells (~58% decrease; $p < 0.01$, data not shown) than in the number of mesenchymal cells (~47% decrease; $p < 0.05$, data not shown). These results suggest that *RUNX2* is part of the signaling pathway that controls EMT initiation (activation of endothelial cells).

RUNX2-I inhibition alters markers of EMT

To confirm chicken AVC EMT is being disrupted after *RUNX2* knock down, expression of smooth muscle alpha actin (α SMA) (Boyer et al., 1999b), Collagen 1 (COL1) (de Vlaming et al., 2012) and Periostin (POSTN) (Inai et al., 2008) messages were assessed by qPCR (Fig. 4H). There was a significant downregulation of α SMA (~35% decrease, $p < 0.001$) and *POSTN* (~60% decrease, $p < 0.01$). *COL1* was not significantly affected ($p=0.275$). These results show that *RUNX2* is required for proper EMT in vitro.

Snai2 and RUNX2-I regulate different aspects of EMT activation

To further our analysis of *RUNX2* function in early (activation) and later (invasion) steps of the chick AVC EMT, the mRNA expression of different EMT markers in control and *RUNX2* siRNA-treated stage 14 and 16 explants were checked by qPCR (Fig. 5A-B). In stage 14 explants cultured for 16 hours (Fig. 5A), *RUNX2* downregulation (~30%, $p < 0.01$) caused significant decrease in the zinc-finger transcription factors *SNAI1* (~47% decrease, $p < 0.001$), *ZEB1* (~42% decrease, $p < 0.001$) and *ZEB2* (~10% decrease, $p < 0.01$) while *SNAI2* showed no significant change ($p=0.307$). The bHLH transcription factor *TWIST1* was decreased (~34% decrease, $p < 0.001$) in transfected explants as well. Additionally, a significant decrease in endothelial VEGF receptors *FLK-1(VEGFR2)* (~70% decrease, $p < 0.001$) and *FLT-1(VEGFR1)* (~40% decrease, $p < 0.001$) was observed in transfected explants. In stage 16 explants cultured for 16 hours (Fig. 5B), *RUNX2* downregulation (~25%, $p < 0.001$) caused a significant decrease in *SNAI2* (~25% decrease, $p < 0.05$) and *ZEB1* (~10%, $p < 0.05$) while *SNAI1* ($p=0.434$) and *ZEB2* ($p=0.316$) were not significantly affected. *TWIST1* was significantly increased (~1.25-fold, $p < 0.05$). Finally, *FLK-1*, but not *FLT-1* ($p=0.203$), was affected (~27% decrease, $p < 0.001$) after *RUNX2* knock down. This molecular analysis confirms *RUNX2* is mainly functioning in the activation step of EMT.

Next, the changes in *RUNX2* siRNA-transfected explants were compared to changes in *SNAI2* siRNA-transfected explants (Fig. 5C-D). *SNAI2* is a known mediator of TGF β 2 signaling in the activation step of the chick heart EMT (Romano and Runyan, 1999; Romano

and Runyan, 2000). *SNAI2* siRNA-transfected stage 14 explants (Fig. 5C, ~25% decrease in *SNAI2*, $p < 0.001$) presented a significant decrease in *ZEB1* (~25% decrease, $p < 0.001$) and *ZEB2* (~40% decrease, $p < 0.001$) while *SNAI1* expression was increased (~1.4-fold increase, $p < 0.01$). *RUNX2* ($p=0.053$), *TWIST1* ($p=0.431$), *FLK-1* ($p=0.408$) and *FLT-1* ($p=0.395$) were unchanged. *SNAI2* knock down (~40% decrease, $p < 0.001$) in stage 16 transfected explants caused significant changes in *ZEB2* (~30% decrease, $p < 0.001$), *TWIST1* (~1.4-fold increase, $p < 0.01$) and *FLK-1* (~1.5-fold increase, $p < 0.001$). Other markers were unchanged (*RUNX2*, $p=0.061$; *ZEB1*, $p=0.172$; *SNAI*, $p=0.683$; *FLT-1*, $p=0.193$). Taken together, these data argue that *RUNX2* functions independently of *SNAI2* in the chicken AVC EMT activation step.

RUNX2 expression in the progression of esophageal carcinoma is consistent with an early role in pathological EMT

RUNX2 is expressed in breast, prostate and thyroid cancers, functioning to promote EMT and invasion (Baniwal et al., 2010; Chinge et al., 2011; Mendoza-Villanueva et al., 2011; Niu et al., 2012). Because esophageal adenocarcinomas are reported to involve TGF β signaling (Onwuegbusi et al., 2006; von Rahden et al., 2006), *RUNX2* expression was examined in staged human esophageal tissues in the transition from normal squamous epithelium (Fig. 6A) to metastatic esophageal adenocarcinoma (Fig. 6D). *RUNX2* expression appears coincident with the transition from columnar epithelia observed in Barrett's Esophagus (Fig. 6B) to high-grade dysplasia (Fig. 6C). *RUNX2* expression persisted in esophageal adenocarcinoma samples (Fig. 6D), suggesting a role for *RUNX2* in EMT and invasiveness.

DISCUSSION

While it is well-known *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. A report showing the expression of *RUNX2* in mesenchymal cells of the AV canal in the developing mouse heart was intriguing (Gitler et al., 2003). Based upon communication with the original provider of the published probe, the initial report likely showed localization of *POSTN* rather than *RUNX2*. *POSTN* (periostin) was previously named OSF-2 while *RUNX2* was OSF2 and periostin was shown to be expressed in cardiac mesenchymal cells (Kruzynska-Frejtag et al., 2001). However, we found expression of authentic *RUNX2* in the chick by rtPCR (Tavares et al., 2006; Mercado-Pimentel and Runyan, 2007). These data prompted us to explore *RUNX2*'s role in embryonic heart development.

Our current model of EMT by embryonic cardiac endothelia divides the process into at least two separate steps. The first step is an activation process by an extracellular stimulus. This activation stage is characterized by cellular hypertrophy and cell separation (Markwald et al., 1977), a change in cellular polarization (Krug et al., 1985), and an intracellular calcium flux (Runyan et al., 1990). As shown by experiments that block activation, this stage is induced, at least in part, by TGF β 2 and mediated by TBRIII (Brown et al., 1999; Boyer and Runyan,

2001). The SNAI2 transcription factor appears to be involved in EMT at this stage (Romano and Runyan, 2000). Staining data in Figure 3 show that both pSMAD 1/5/8 and pSMAD 2/3 appear to be active at this stage of heart development. The data show that RUNX2-1 expression is unaffected by BMP-2 or Noggin signaling. We note that the ALK-2 receptor is active during cardiac EMT and that ALK-2 binds both TGF β and BMP ligands and can signal via SMAD1/5 (Mercado-Pimentel and Runyan, 2007; Derynck and Feng, 1998; Daly et al., 2008). The staining pattern may indicate that TGF β is signaling via both the SMAD 2/3 and SMAD 1/5/8 pathways.

A separate and second step of EMT is characterized by invasion of mesenchymal cells derived from activated endothelial cells into the underlying matrix. Inhibition of TGF β 3 (Potts et al., 1991; Boyer et al., 1999a), TBRII (Brown et al., 1996; Boyer and Runyan, 2001), ADGRL2 (LPHN2, (Doyle et al., 2006), and Olfactomedin-1 (Lencinas et al., 2013) all show normal activation but a loss of cell invasion by explanted tissues in vitro. The invasion step requires elements of cytoskeletal change, alteration of cell adhesion, expression of proteases and secretion of extracellular matrix proteins whose coordination is unclear (Person et al., 2005; Combs and Yutzey, 2009). While all atrioventricular canal endothelial cells appear to be activated, only 7–10% of the cells complete EMT and become invasive. We suggest that completion of EMT may require passage through one or more checkpoints and that incomplete EMT may account for the concept of epithelial plasticity in EMT (Lencinas et al., 2013).

Our present findings indicate that RUNX2-I is the only RUNX2 isoform present in the embryonic heart at the time of EMT and is required for this process to properly initiate after TGF β 2 induction (Fig. 7), as seen in RUNX2 siRNA treated AV canals. The observation that *RUNX2-I* is significantly down-regulated when treated with anti-TGF β 2, but not by anti-TGF β 3, also supports that *RUNX2-I* expression is correlated with the activation step of EMT rather than the invasion step (Boyer et al., 1999a). This is also consistent with the loss of normal cell-cell separation observed with the activation step of EMT (Figure 3). When TGF β 3 is added to cultures we see a small increase in *RUNX2* mRNA expression, but it is not as strong as when TGF β 2 is added. It is known that differences in potency of exogenous TGF β s can reflect isoform specificity in vivo but that all isoforms can produce a response in vitro (Roberts et al., 1990). These data suggest that, in the heart, TGF β 2 is the principal mediator of *RUNX2-I* expression.

Downregulation of *RUNX2-I* and reduced activation of AV canal endothelia after treatment with RUNX2 siRNA show that this factor is required for proper EMT. Quantification of activated endothelial cell and mesenchymal cell numbers confirmed this requirement. The observation that EMT was not completely blocked and a few mesenchymal cells could be observed invading the gel in treated explants can be explained by either compensation by other factors or, more likely, by the observation that siRNA treatments reduce mRNA levels with variable efficiency and may cause partial phenotypes (Kurreck, 2006). Though we observed somewhat variable levels of *RUNX2* mRNA downregulation between experiments, this downregulation always disrupted EMT and produced changes in marker gene expression. qPCR analysis of selected EMT markers showed downregulation of *α SMA* and *POSTN*. Expression of α SMA has been a consistent marker of activation and mesenchymal

cell formation. Its expression is detected in endothelial cells after EMT activation (Boyer et al., 1999b) and in endothelial tissues treated with high levels (20 ng/ml) of TGF β 3 (Nakajima et al., 1997). Periostin is an extracellular protein expressed in valvular mesenchymal cells and by metastatic cancer cells (Kruzynska-Frejtag et al., 2001; Inai et al., 2008; Liu et al., 2015). Collagen I is a well-known extracellular matrix component secreted by newly formed mesenchymal cells but is also expressed by embryonic cardiomyocytes during the formation of the cardiac cushions (Sinning et al., 1988; de Vlaming et al., 2012; Hosper et al., 2013). The decrease in *α SMA* and *POSTN* levels after *RUNX2* downregulation is consistent with the role of these markers in EMT while the lack of significant change in Collagen I may reflect the larger continued synthesis of ECM by myocardial cells.

The finding *RUNX2-I* is induced by TGF β 2 and therefore functions during EMT initiation led us to investigate changes of EMT markers after *RUNX2-I* knock down in cultured stage 14 (during EMT initiation) and stage 16 (EMT invasion) explants. Previous in vitro experiments in this lab show that activation elements of EMT predominate in analysis explants collected from stage 14 or 15 embryos and cultured for 24 hr or less. In contrast, invasion components of EMT are predominant in explants derived from stage 16 and 17 chicks and cultured overnight or longer (Potts and Runyan, 1989, Boyer et al., 1999a; Mercado et al., 2006; Lencinas et al., 2013). Zinc-finger and bHLH transcription factors are upregulated in various EMT models and their expression correlates with downregulation of E-cadherin or VE-cadherin prior to cell delamination (Romano and Runyan, 1999; Bolos et al., 2003; Vandewalle et al., 2009; Montserrat et al., 2011). Therefore, EMT inhibition could potentially cause downregulation of these factors. *RUNX2* inhibition in stage 14 explants caused downregulation of four EMT-related transcription factors (*SNAI1*, *ZEB1*, *ZEB2* and *TWIST1*), whereas inhibition in stage 16 caused downregulation only of *SNAI2* and *ZEB1*. The stronger inhibition at the earlier stage of EMT is consistent with a role for *RUNX2-I* in EMT initiation or activation (around stage 14) by TGF β 2 (Boyer et al., 1999a).

That *SNAI2* is also a transcription factor that is TGF β 2-regulated and required for proper EMT (Romano and Runyan, 1999; Romano and Runyan, 2000), led us to compare the effects of *RUNX2-I* downregulation to *SNAI2* downregulation. We observed that these two transcription factors are regulating different aspects of EMT after TGF β 2 induction. As expected, *SNAI2* knockdown caused changes that were more correlated to EMT inhibition (*ZEB1* and *ZEB2* downregulation) in stage 14 explants (when activation is predominant) rather than in stage 16 explants (when invasion is predominant). This is consistent with both the knockdown of *RUNX2-I* observed here and *SNAI2*'s role in the activation step (Romano and Runyan, 1999; Romano and Runyan, 2000). We observed an increase in *SNAI1* after *SNAI2* downregulation in stage 14 explants while *RUNX2-I* knockdown caused a decrease in *SNAI1* levels. Additionally, *TWIST1* was downregulated after *RUNX2-I* inhibition in stage 14 explants but upregulated after both *RUNX2-I* and *SNAI2* knockdown in stage 16 explants. This likely represents a change in *TWIST1* regulation after EMT has been initiated in activated endothelia and mesenchymal cells are invading. Thus, while many of the EMT-associated transcription factors are found in the heart, the interactions between them clearly need more examination.

The most drastic effect was observed in the levels of *FLK-1 (VEGR2)* and *FLT-1 (VEGR1)*, endothelial markers shown to be up-regulated and down-regulated, respectively, in response to EMT (Stankunas et al., 2010). *RUNX2* siRNA treatment of stage 14 explants caused downregulation of both molecules whereas *SNAI2* siRNA treatment produced no changes at the same stage. *FLK-1* downregulation continued in stage 16 explants after *RUNX2* inhibition showing that *RUNX2* is required for induction of this receptor during both activation and invasion steps of EMT. Additionally, *SNAI2* inhibition in stage 16 explants caused upregulation of *FLK-1*. Finally, while *FLK-1* is behaving in agreement with the pattern previously described, *FLT-1* is not (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 as markers of the transition to mesenchyme.

The role of *RUNX2* in EMT is not widely explored but may be conserved in both developmental and metastatic EMTs. A developmental role for an ortholog of *RUNX2*, Zebrafish *RUNX2a*, is suggested by its localization in neural crest and somites of early embryos, both sites of EMT (Flores et al. 2004). Expression of *RUNX2* during the progression from Barrett's esophagus to dysplasia is consistent with the idea that *RUNX2-I* may have a common role in the EMT in both metastasis and development at an early or "activation" stage. Progression of esophageal cancer is mediated by *TGFβ* (von Rahden et al., 2006). Barrett's esophagus is a metaplasia of the lower esophagus associated with reflux that can progress (0.5% per patient year) to dysplasia and adenocarcinoma. *RUNX2* staining is observed in dysplasia and thus appears to be associated with an early stage of cancer progression and may be similarly regulated by *TGFβ* expression in the tissue. Though not tied to *TGFβ* signaling, loss of *RUNX2* in thyroid carcinoma was associated with loss of a broad spectrum of EMT markers (Niu et al., 2012). *RUNX2* expression appears to be linked to a variety of cancers (Voon and Thiery, 2017).

In summary, we find the 2-I isoform of *RUNX2* to be specifically expressed in the developing heart and show that it has a role in the activation stage of endothelial EMT as loss of expression inhibits the cell separation characteristic of this stage. It appears to be regulated by the *TGFβ2* isoform that is also associated with activation of EMT in the heart. Though it is not clear whether they are direct or indirect targets, loss of *RUNX2-I* inhibits the expression of the mesenchymal markers, *POSTN* and *α-SMA*. Though coincident in expression and regulation by *TGFβ2*, *RUNX2-I* is not redundant with *SNAI2* in its regulation of the various EMT markers and the activation of EMT. Though *Runx2* has been previously observed in cancer, an isoform-specific function of *RUNX2* in EMT suggests that this isoform may be linked to metastatic EMT rather than the subsequent progression of cancer to bone tissue.

EXPERIMENTAL PROCEDURES

Embryos

Fertilized White Leghorn eggs were obtained from Texas A & M University, Department of Poultry Sciences and incubated at 37.5°C.

Immunofluorescent Microscopy

Chicken embryos were harvested in Tyrode's solution at stages 14 to 20 (Hamburger and Hamilton, 1951) and at embryonic day (E)13. The heart region and forelimb of collected embryos were dissected and fixed in solution of -70°C 80% methanol / 20% DMSO and cryosubstituted at -20°C for at least one week. Tissues were then embedded in paraffin and sectioned at $7\mu\text{m}$. Sections containing the AV canal region of the heart were processed using an indirect immunofluorescence technique. A mouse monoclonal primary antibody against recombinant RUNX2 was used (Santa Cruz Biotechnology). Specificity of this antibody against chick RUNX2 was tested by western blot (data not shown). Briefly, after deparaffinization, the sections were rinsed in 1X PBS for 10 min, blocked for 1 hour at room temperature with a blocking solution containing 1% bovine serum albumin and 0.1% Tween 20 in PBS and incubated overnight with the primary antibodies at -4°C in a moist chamber. After several rinses in 1X PBS, Alexa fluor 488 or 546-conjugated rabbit anti-mouse secondary antibody (ThermoFisher Scientific) were incubated for 1 hour at room temperature in moist chamber. After rinsing in PBS, the nuclei were stained with TO-PRO-3 (ThermoFisher Scientific) and the sections mounted using Prolong Gold mounting media (ThermoFisher Scientific). The sections were analyzed using a LSM 510 Zeiss Confocal Microscope.

For the in vitro localization of RUNX2 and pSMADs, AVC endocardial cushion explants of stage 17 chicken embryos were collected and incubated for 24 hours (37°C , 5% CO_2) with complete medium 199 (1X Medium 199 (Sigma-Aldrich) + Antibiotic/Antimycotic (ThermoFisher Scientific) + ITS Premix (5 $\mu\text{g}/\text{ml}$ insulin, 5 $\mu\text{g}/\text{ml}$ transferrin, and 5 $\mu\text{g}/\text{ml}$ selenium – ThermoFisher Scientific) on a type I collagen gel as described previously (Potts and Runyan, 1989). Gels were fixed with 4% paraformaldehyde for 25 min at room temperature, rinsed with 1X PBS for 1 hour at room temperature and processed for RUNX2 and pSmad1/5/8 or pSmad2/3 immunofluorescence microscopy with the antibodies described above and rabbit polyclonal anti-pSmad1/5/8 (Abcam) and rabbit polyclonal anti-pSmad2/3 (Abcam). Nuclei were stained with DAPI (Sigma-Aldrich) and F-actin with Alexa Fluor 488 or 647 phalloidin (ThermoFisher Scientific). Gels were analyzed using a DeltaVision Deconvolution Microscope.

RNA isolation, RT-PCR and Quantitative Real-Time PCR analyses

Total RNA was extracted from isolated E13 chick wings, stage 17 chick embryos, microdissected stage 17 hearts or from incubated explants on gels using the PureLink RNA Mini kit (ThermoFisher Scientific). 1 μg of RNA was used from samples to make cDNA using the qScript cDNA Synthesis kit (Quanta Biosciences). Semi-quantitative analysis of *RUNX2* isoforms was performed by PCR amplification (30 cycles) using primers designed to specifically identify type I *RUNX2* (FW: 5'-TGC GTATTCCCGTAGATCC-3'; RV: 5'-TTAGGAGAAGTGCCCGATGGGA-3') or type II *RUNX2*: (FW: 5'-GCATCAAACAGCCTCTTCAGCTC-3'; RV: 5'-TTAGGAGAAGTGCCCGATGGGA-3'). *GAPDH* was used as loading control. PCR products were analyzed on 1% agarose gels. Quantitative Real-time PCR was performed using the FastStart SYBR Green Master (Sigma-Aldrich) on RotorGene 3000 PCR machine (Corbett Research). Equal amounts of cDNA were added for each reaction, and in each experiment triplicate reactions were performed for

each gene to be analyzed. Quantitative data were normalized with HPRT and β -actin. Experiments were repeated three times and results analyzed with Rotor Gene 6 software (Corbett Research) using the comparative quantitation method and Student's *t* Test. Primer sequences used in these experiments are shown in Table 1.

Whole-mount in situ hybridization

Stage 18 chick embryos were fixed in 4% PFA overnight at 4°C. Embryos were processed for hybridization per (Nieto et al. (1996) with minor modifications. Digoxigenin-labelled RNA probe was synthesized with T3 RNA polymerase (Sigma-Aldrich) and DIG RNA labeling mix (Sigma-Aldrich). Probe template (434nt) was amplified by PCR with the following primers: FW: 5'-TTATAAAAGCTTGCGCCGCAGAATAT-GCTGCGAAATGCTTCTGCTG-3'; RV: 5'-GCTCTAGAAATTAACCCTCACTAAAGG-AAGTCATCTGGCTCAAGTAGGACG-3'

In vitro inhibition and stimulation of TGF β family members

Neutralizing anti-porcine TGF β 2 (20 μ g/ml, goat IgG, R&D Systems), anti-TGF β 3 (20 μ g/ml, mouse IgM, Developmental Studies Hybridoma Bank) antibodies, recombinant human Noggin (50ng/ml), porcine TGF β 2 (0.002ng/ μ l), recombinant human TGF β 3 (0.002ng/ μ l), recombinant human BMP-2 (0.002ng/ μ l) (R&D Systems) were diluted in complete medium. Gels were pre-treated with each solution prior to explantation. Control gels were pre-treated with complete medium alone or complete medium + IgG/IgM. Stage 14 AVC explants were dissected and collected in 1X Medium 199. The explants were placed on the pre-treated gels and incubated overnight for adhesion (37°C, 5% CO₂). More treatment was added to gels the following morning and cultures were incubated for a total of 24 hours after the explants were initially placed on the gel. Total RNA was extracted as described above. The samples were then processed for Quantitative Real-Time PCR.

Inhibition of *RUNX2* and *SNAI2* message by siRNA knockdown

Small interfering RNAs (siRNAs) homologous to the 3'ORF of *RUNX2* (NM_204128) were designed and synthesized using the Silencer siRNA Construction Kit (ThermoFisher Scientific) or purchased from ThermoFisher Scientific (Silencer Select Custom Designed siRNA). Silencer Select Negative Control was also purchased from ThermoFisher Scientific. The sequences of *RUNX2* siRNA templates were: Silencer Select Custom Designed siRNA: GAGCTTTACTTTGACAATA; Silencer siRNA-1: GGACAGAGTCAGATTACAG; Silencer siRNA-2: TTCTAGTGGCAGGATGGAT; *SNAI2* siRNA template was: TCTAGGAAATCGTTCAGCTGCAAGTACTGT. siRNAs were diluted in 1X Medium 199 to a concentration of 50nM and transfected using the siPORT NeoFX Transfection Agent (ThermoFisher Scientific). Briefly, stage 14 or 16 explants were collected in 1X Medium 199 and added to medium containing siRNAs. They were incubated for 1h 30 min at 37°C, 5% CO₂ and then placed on gels. After an adhesion period (overnight, 37°C, 5% CO₂), new transfection solution was added to cultures and the incubation allowed to continue for up to 16h, 24h or 48 hours after the explants were initially placed on the gel. The gels were either processed for RNA extraction as described above or fixed with 4% paraformaldehyde for 25 min at room temperature and endothelial outgrowth from the explant and mesenchymal cell invasion of the collagen gel were qualitatively and quantitatively analyzed using Hoffman

Modulation contrast optics. Activated cells, defined as isolated cells on the surface of the gel, and mesenchymal cells defined as cells wholly within the gel matrix, were counted. A total of 14 explants (7 control explants, 7 experimental explants) were examined and the data analyzed with Student's t test.

Immunohistochemistry

Samples from control patients, patients with Barrett's esophagus, with high-grade dysplasia or with esophageal adenocarcinoma were obtained from the Biobank core at the Cancer Center at the University of Arizona, Tucson – AZ. They were fixed in 4% PFA, paraffin embedded and processed for RUNX2 immunohistochemistry using a mouse monoclonal primary antibody against recombinant RUNX2 (Santa Cruz Biotechnology). Detection was performed with an HRP-conjugated anti-mouse secondary antibody (ThermoFisher Scientific) and DAB (ThermoFisher Scientific). Sections were counterstained with H&E. Samples from 30 patients for each group were analyzed for RUNX2 expression.

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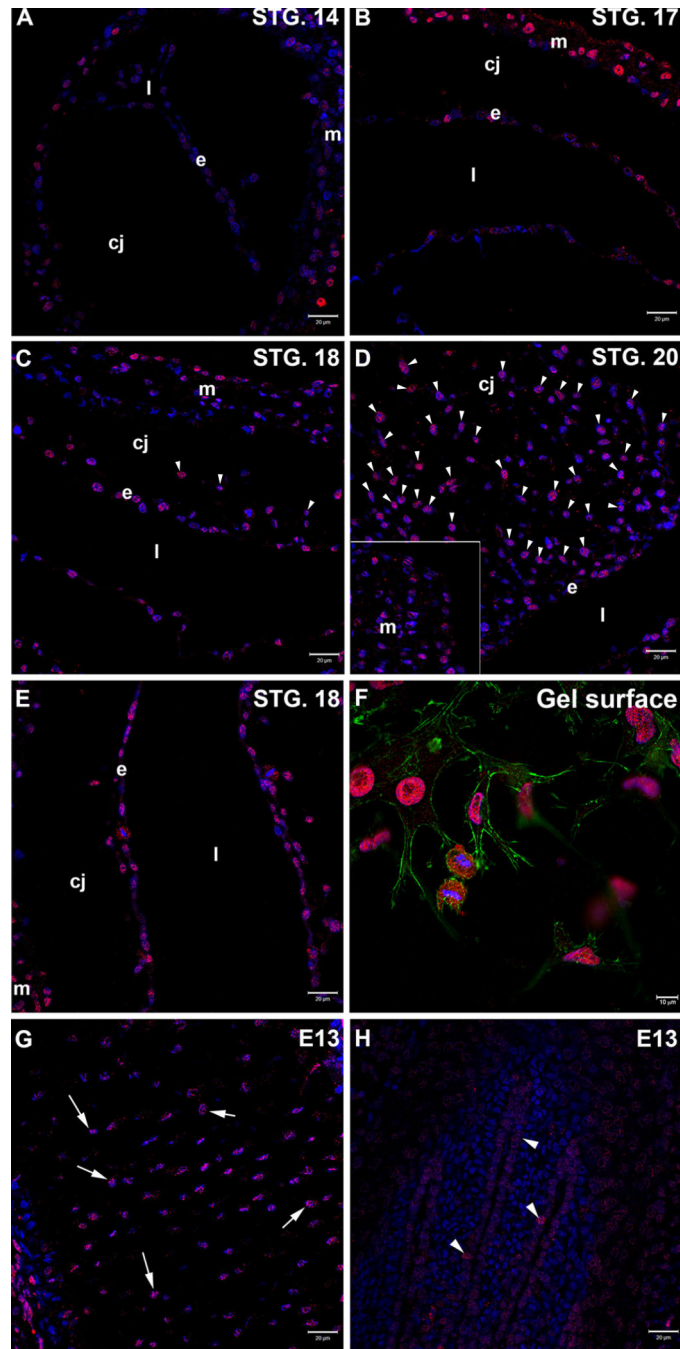


Figure 1. RUNX2 immunolocalization in the chick embryo heart during the timing for EMT A-E. Chicken embryo hearts were stained for RUNX2 (red) in the AVC region (A-D) or OFT (E). Nuclei were counterstained with TO-PRO-3 (blue). In the AVC region of stage 14 chicken hearts (A), low expression of RUNX2 is detected in endothelial cells (e) lining the heart lumen (l). This expression is increased by stage 17 (B) and stage 18 (C). RUNX2 is still detected in endothelial cells (e) by stage 20 (D). Mesenchymal cells (arrowheads) invading the heart cardiac jelly (cj) show expression of RUNX2 in stage 18 (C) and stage 20 (D) hearts. A similar expression pattern is observed in stage 18 OFT region (E). RUNX2 is

also present in the developing myocardium (m) at all analyzed stages (A-C, inset in D, E). Scale bar: 20µm. **F.** Activated endothelial cells derived from cultured AVCs on a collagen gel lattice show RUNX2 expression (red) in the nuclei. Low RUNX2 level is also detected in the cytoplasm. Nuclei were counterstained with DAPI (blue) and F-actin with phalloidin (green). Scale bar: 10µm. **G-H.** E13 chicken wings were stained for RUNX2 (red) as positive controls. The antibody marks osteoblasts in a region of endochondral ossification (arrows in G) and cells in the feather papilla (arrowheads in H). Nuclei were counterstained with TO-PRO-3 (blue). Scale bar: 20µm.

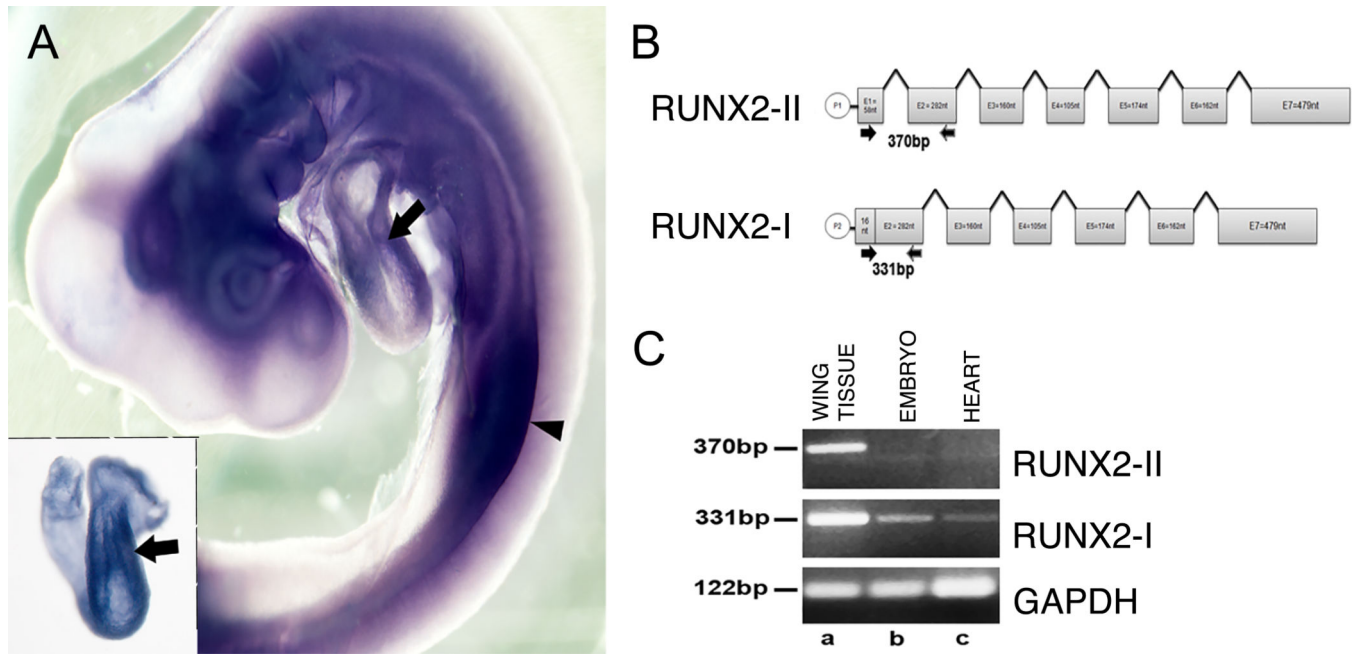


Figure 2. RUNX2-I is the isoform present in the chick heart during EMT

A. Lateral view of a stage 18 chicken embryo processed for whole-mount in situ hybridization using a probe recognizing chicken RUNX2. There is expression of RUNX2 in the limb buds (arrowhead) and in the heart, including the AVC region (arrows). The inset shows a dissect heart for better visualization. **B.** Schematic representation of RUNX2 gene. Transcription of RUNX2-II, the bone specific isoform, starts at the distal promoter P1. Transcription of RUNX2-I starts at the proximal promoter P2. RUNX2-II was amplified using primers that produced a 370bp product whereas RUNX2-I was amplified using primers that produced a 331bp product. **C.** RT-PCR analysis of total RNA from E13 chick wing tissue (a), stage 17 embryos (b) and stage 17 hearts (c) shows RUNX2-II (top lane) is expressed only in E13 chicken wing cells (a) while RUNX2-I (middle lane) is expressed in chicken wing cells (a), stage 17 embryos (b) and stage 17 hearts (c). Bottom lane shows GAPDH expression (122bp) as loading control.

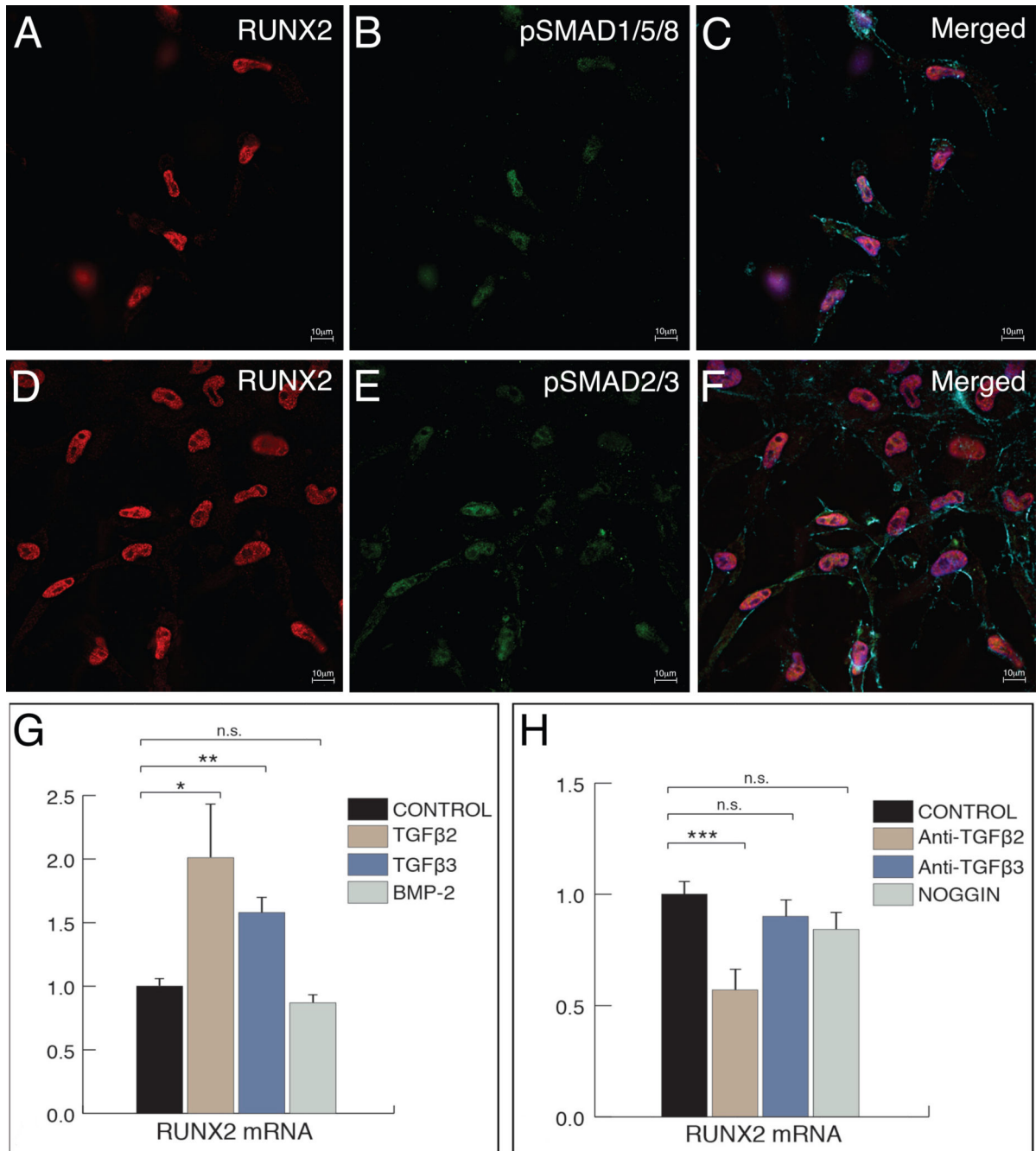


Figure 3. RUNX2-I is induced by TGFβ signaling

A-F. Activated endothelial cells derived from cultured AVC explants co-express RUNX2 (red in A and D) and pSmad1/5/8 (green in B) or pSmad2/3 (green in E). Merged picture in C and F confirms co-localization of the proteins in the cell nuclei (DAPI, blue). F-actin was stained with phalloidin to show cell morphology (cyan). Scale bar: 10μm. **G.** qPCR analysis of RUNX2 mRNA in AVC explants cultured in the presence of TGFβ2, TGFβ3 or BMP-2. Addition of TGFβ2 or TGFβ3 to culture media caused a significant increase in RUNX2 message levels whereas addition of BMP-2 caused no significant changes. **H.** qPCR analysis

of RUNX2 mRNA in AVC explants cultured in the presence of neutralizing antibodies against TGF β 2 or TGF β 3 or with the BMP inhibitor, noggin. Blockage of TGF β 2 caused a significant decrease in the level of RUNX2 mRNA whereas blockage of TGF β 3 or BMP signaling did not cause any significant changes. Error bars = S.E.M.; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. – non-significant.

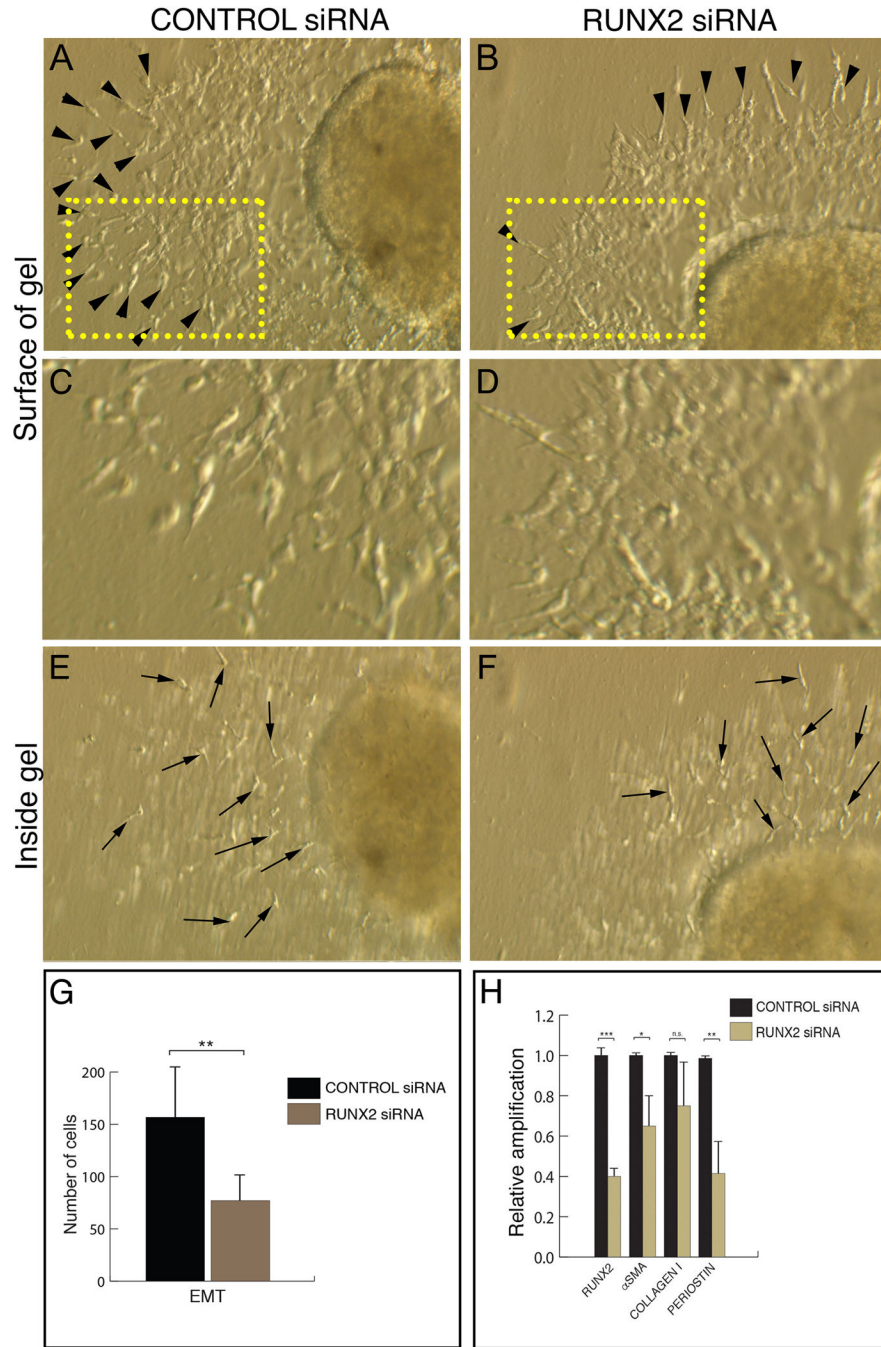


Figure 4. RUNX2-I is required for the activation step of chick heart AVC EMT

A-F. Stage 14 chicken AVC explants cultured for 24h in the presence of a scrambled siRNA (A, C, E) or a siRNA against RUNX2 (B, D, F). In control explants, multiple activated endothelial cells (arrowheads in A) are present on the gel surface. C represents a higher magnification of the boxed area in A showing the endothelial cell outgrowth. Inside the gel, multiple invasive mesenchymal cells (arrows in E) are observed. RUNX2 siRNA-transfected explants show fewer activated endothelial cells (arrowheads in B) on the gel surface. D represents a higher magnification of the boxed area in B showing a compact endothelial cell

outgrowth. Mesenchymal cells (arrows in F) are present in treated explants. **G.** Quantitation of the number of EMT-derived cells in control and RUNX2 siRNA-transfected explants. There is a significant decrease in the number of activated endothelial cells and mesenchymal cells in treated explants compared to control explants (Average cell number in control explants = 156.71 ± 18.83 ; average cell number in RUNX2 siRNA-treated explants = 77.00 ± 9.07 ; ~51% decrease, $p < 0.01$). $n=14$ (7, control; 7, experimental). **H.** qPCR analysis of RUNX2 and mesenchymal cell markers in control and RUNX2 siRNA-transfected explants. There is a significant decrease (~60%) in RUNX2 mRNA after siRNA treatment. RUNX2 downregulation is followed by a decrease in the levels of α SMA (~35% decrease) and PERIOSTIN (POSTN) (~60% decrease). COLLAGEN I is unchanged in treated explants. Error bars = S.E.M.; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. – non-significant.

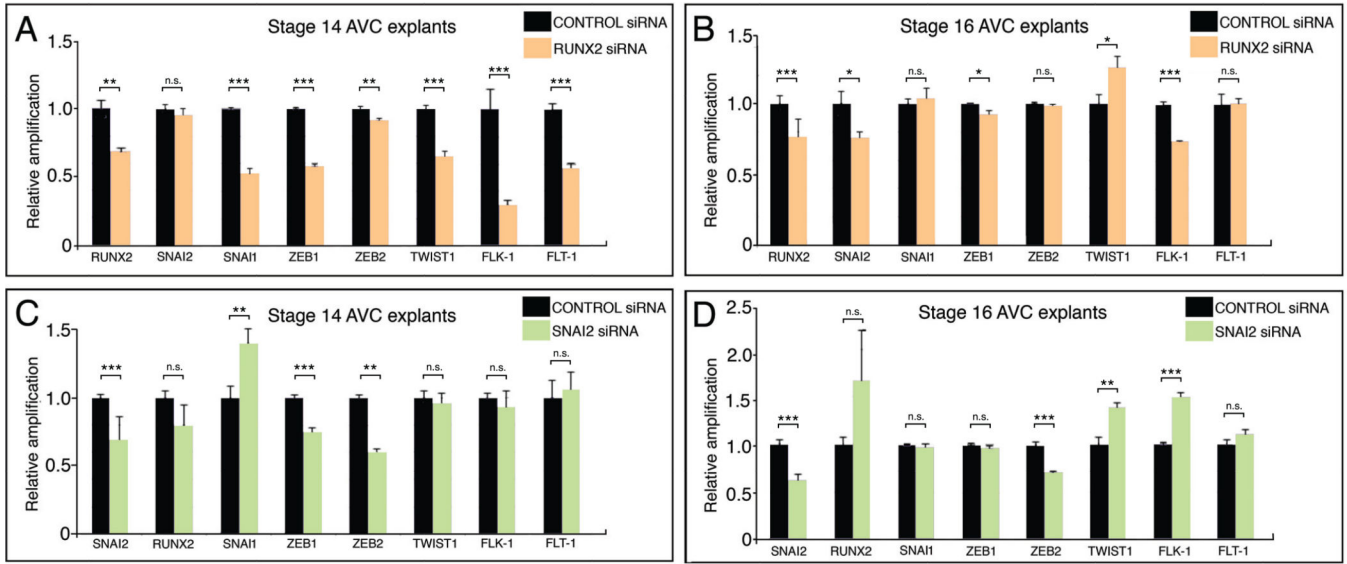


Figure 5. RUNX2-I regulates EMT independently of SNAI2

qPCR analysis of EMT-related transcription factors and receptors in stage 14 (A, C) and 16 (B, D) control and RUNX2 siRNA (A-B) and SNAI2 siRNA (C-D) transfected explants cultured for 16h. **A.** In cultured stage 14 explants, RUNX2 downregulation (~30%) caused a significant decrease in SNAI1 (~47%), ZEB1 (~42%), ZEB2 (~10%), Twist1 (~34%), FLK-1 (~70%) and FLT-1 (~40%). SNAI2 was not significantly changed in treated explants. **B.** In cultured stage 16 explants, RUNX2 downregulation (~25%) caused a significant decrease in SNAI2 (~25%), ZEB1 (~10%) and FLK-1 (~27%) and a significant increase in TWIST1 (~1.25-fold increase). All other markers did not show significant changes. **C.** In cultured stage 14 explants, SNAI2 downregulation (~25%) produced a significant decrease in the levels of ZEB1 (~25%) and ZEB2 (~40%). SNAI1 was significantly increased after treatment (~1.4 fold). All other genes were unchanged in treated explants. **D.** In cultured stage 16 explants, SNAI2 downregulation (~40%) caused a significant decrease in ZEB2 (~30%) and a significant increase in TWIST1 (~1.4-fold) and FLK-1 (~1.5-fold). All other markers did not show significant changes. Error bars = S.E.M.; * p < 0.05; ** p < 0.01; *** p < 0.001; n.s. – non-significant.

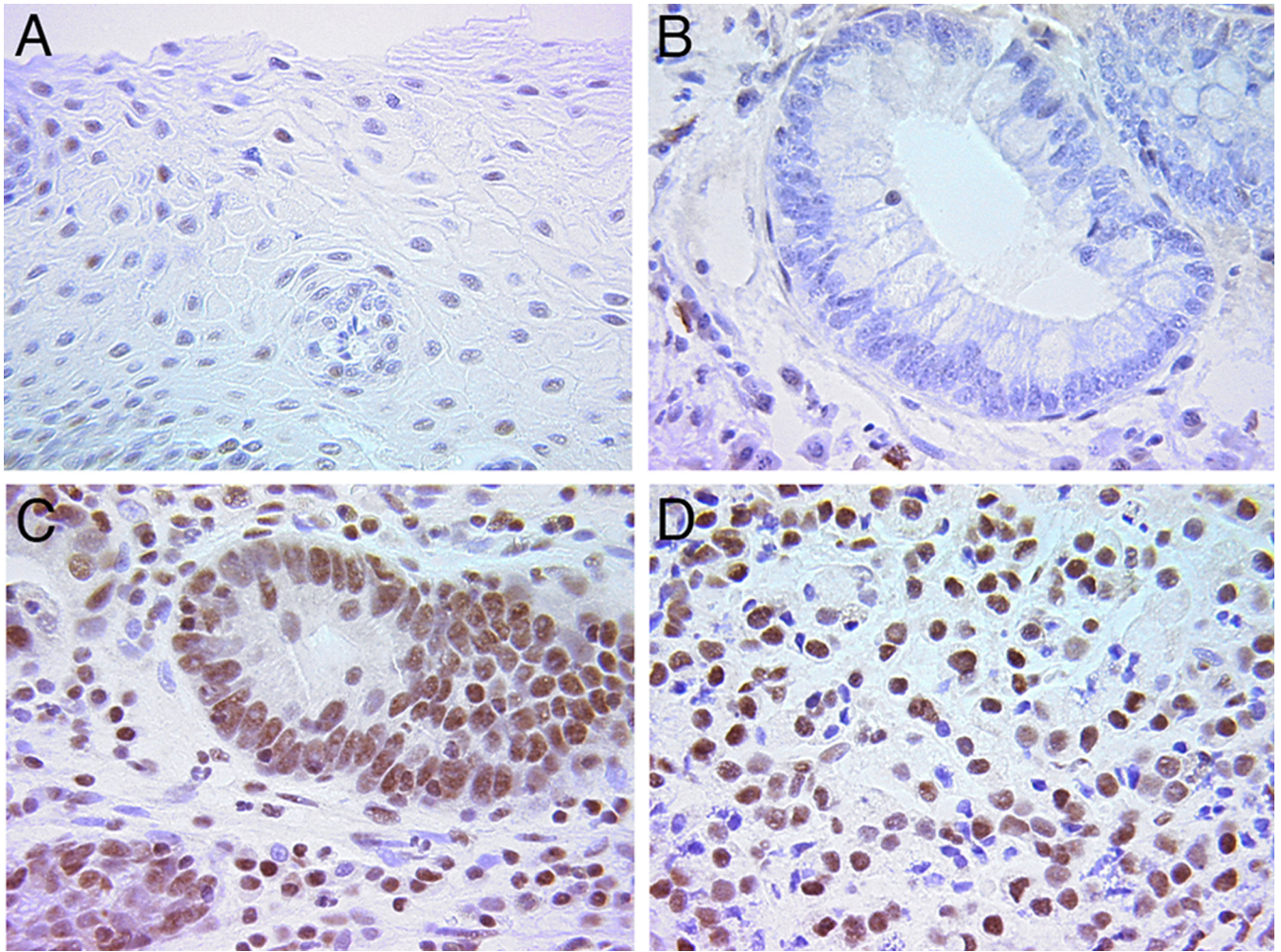


Figure 6. Expression of RUNX2 in staged esophageal adenocarcinoma materials

A. RUNX2 is not expressed in normal esophageal squamous epithelium from a control patient. **B.** Columnar esophageal epithelium from a patient with Barrett's Esophagus does not show RUNX2 expression. **C.** RUNX2 is highly expressed in esophageal tissue from a patient with high-grade dysplasia. **D.** Esophageal adenocarcinoma continues to express high levels of RUNX2. RUNX2 positive staining: brown color. Sections were counterstained with H&E.

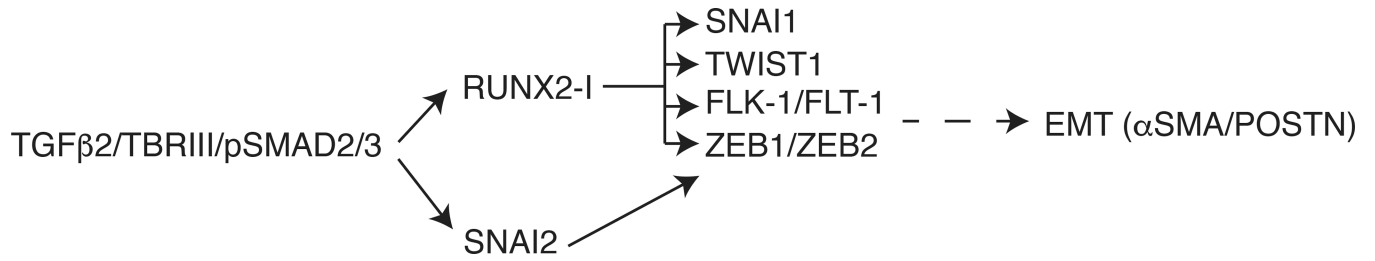


Figure 7. RUNX2-I functions in the chick AVC EMT activation step

In the chick heart AVC, TGFβ2 binds to TBRIII expressed by endothelial cells. This signaling pathway through pSMAD2/3 turns on the expression of RUNX2-I and SNAI2 (Romano and Runyan, 2000), two factors required for EMT activation. RUNX2-I induces the expression of EMT-related transcription factors and VEGF receptors independently of SNAI2 with the exception of ZEB1 and ZEB2. Expression of these molecules is followed by delamination of endothelial cells and expression of αSMA and POSTN.

Table 1

qPCR primers used for measurement of EMT markers

Primer	Forward	Backward
αSMA	5'-AGGACAGCACTGCCCTTGTTT-3'	5'-CCCATACCAACCATCACACCCT-3'
β-actin	5'-CCCCGTGCTGTGTTCCCATCTATCG-3'	5'-TGATTTTCATTGTGCTAGGTGCCAGG-3'
COL1a1	5'-TGTGGATTCTCGTTACTGCTGTT-3'	5'-ATCTGGCACGGTTCGGGTTT-3'
FLK-1	5'-CAGCCCTGCGCCTCTTGTGT-3'	5'-GGCAAGGAAGGCACATCCCGG-3'
FLT-1	5'-CCCAACGAGCGCCATGGTT-3'	5'-CGCAGGGGAAACCCGCTTT-3'
HPRT	5'-TGTTGTTGGATACGCCCTCG-3'	5'-TCTGCTTCCCGTCTCACTG-3'
POSTN	5'-CAGTTGCTACAATCCTGAAGAAGC-3'	5'-TCCCCCTCCCTGACTATTTGAAC-3'
RUNX2	5'-GCGAAATGCTTCTGCTGTGA-3'	5'-AGTCGCTACATAACCACAGAGC-3'
SNAI1	5'-TTGCACTGCGATGCTCAGACCA-3'	5'-TCAGCAAAGCCCGTTGCAAT-3'
SNAI2	5'-GAAAGCCTGCCTTCAAATGC-3'	5'-CCACACAGTAATGGGGTTGTAAGC-3'
TWIST1	5'-TGGTTGTTGCTGTCGTTTTGG-3'	5'-TGGTTGGGTGCTTTGCTTTC-3'
ZEB1	5'-TGACTTGCCAACAGACCACACC-3'	5'-CTTTTCCTTCATCTCCAGCAG-3'
ZEB2	5'-GCCTTGAAAAACCTTTGCTACCTG-3'	5'TTGCTCTTCGTTCTTGTTCCTC-3'