Homeobox, Wnt and fibroblast growth factor signaling is augmented during alveogenesis in mice lacking superoxide dismutase 3, extracellular

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Abstract (250 words)

Superoxide dismutase 3, extracellular (SOD3) polymorphisms have been implicated in reduced pulmonary function development and altered risk for chronic obstructive pulmonary disease. We previously reported that gene targeted *Sod3*-/- mice have impaired lung function and human *SOD3* variants are associated with reduced pulmonary function in children. Reduced lung SOD3 levels were reported in mice with lower lung function with the greatest difference occurring during alveogenesis phase [postnatal (P) days 14-28]. Interactions between homeobox (HOX), wingless-type MMTV integration site member (WNT), and fibroblast growth factor (FGF) signaling govern complex developmental processes in several organs. A subset of HOX family members, HOXA5 and HOXB5, are expressed in the developing lung. Therefore in this study we assessed transcript expression of these family members and their downstream targets in *Sod3*-/- mice during alveogenesis (P14). In the lung of *Sod3-/-* mice, *Hoxa5* and *Hoxb5* increased. These transcription factors regulate WNT gene expression and were accompanied by increases in their downstream targets *Wnt2* and *Wnt5A*, canonical and non-canonical WNT members, respectively. The WNT signaling target, lymphoid enhancer binding factor 1 (*Lef1*) also increased along with its downstream targets *Fgf2, Fgf7*, and *Fgf10* in the lungs of *Sod3-/-* mice. Due to limited knowledge on the role of FGF2 in lung development, we further examined FGF2 protein and found increased levels in the bronchial and alveolar type II epithelial cells of *Sod3*-/- mice compared to age-matched control. Thus, our findings suggest that deficient management of extracellular superoxide can lead to altered lung developmental signaling during alveogenesis in mice.

Key words: Superoxide, oxidative stress, lung development, WNT, FGF

Introduction

Failure to attain optimal lung function by early adulthood can be a predisposing factor for the later appearance of chronic lung diseases including asthma and chronic obstructive pulmonary disease [1-3]. In mice, superoxide dismutase 3 (SOD3) can limit lung injury in response to a variety of pulmonary insults [4, 5] and can bind to extracellular matrix components and protect against the formation of bioactive oxidative matrix fragments. In a murine model of bronchopulmonary dysplasia, SOD3 deficiency contributed to hyperoxic lung injury in neonatal mice correlated with alveolar damage [4, 6] Accordingly, previous investigations have focused on whether inherited variation in SOD3 expression and function could influence lung homeostasis during environmental challenges that initiate oxidative injury.

Previously, we reported that JF1/Msf mice have lower lung function and have reduced lung SOD3 transcript, protein, and enzymatic activity compared to C3H/HeJ mice with higher lung function. The greatest difference in SOD3 levels occurring during peak alveogenesis phase [postnatal (P) days 14-28] of lung development [7]. Correspondingly, gene-targeted *Sod3*-/-mice have impaired lung development exhibited by diminished ventilation efficiency [8]. We also reported common *SOD3* genotypes, Ala377Thr [global minor allele frequency (MAF)≈ 40%] and -382C/T (MAF≈ 45%) promoter variant, to be associated with decreased forced expiratory volume in 1 second (FEV_1) and maximum expiratory flow at 25% volume (MEF_{25}) in children [7], further supporting its role in lung function development. Similarly, Dahl et al. [9] identified *SOD3*-3176G/T and -407G/A promoter variants to be associated with reduced FEV_1 and Siedlinski et al. [10] found an Arg213Gly variant to be associated with slower FEV_1 decline in never-smokers and -4466G/T5'-upstream variant to be associated with lower vital capacity.

These findings are consistent with the hypothesis of early developmental origins predisposing chronic lung pathogenesis later in life. However, the mechanism through which SOD3 may contribute to lung function development remains to be explored. Therefore, in this study we assessed the transcript expression of key lung developmental genes in neonatal

Sod3-/- mice and their plausible interactions within signaling pathways during the peak phase of alveogenesis.

Methods

All procedures were approved by Institutional Animal Care and Use Committee of the University of Pittsburgh, PA. *Sod3*-/- (B6.129P2-Sod3^{tm1Mrkl}/J, Stock No: 009654) and *Sod3*+/+ (C57BL/6J, Stock No: 000664) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen free conditions. Food and water were provided ad libitum. Previously we detected a decrease in SOD3 transcripts and protein in JF1/Msf mice, an inbred strain with diminished lung function, as compared to C3H/HeJ mice, an inbred strain with greater lung function. The peak decline was detected at Postnatal day 14 (P14) to P28 during alveogenesis. Therefore, lung transcripts from P14 *Sod3*-/- (n=6) were compared to P14 *Sod3*+/+ (n=5) mice and included: ATP-binding cassette, subfamily A (ABC1), member 3 (ABCA3), axin 2 (AXIN2), disheveled segment polarity protein 3 (DVL3), fibroblast growth factor 2 (FGF2), FGF7, FGF9, FGF10, frizzled class receptor 1 (FZD1), FZD7, homeobox A5 (HOXA5), HOXB5, lymphoid enhancer binding factor 1 (LEF1), NK2 homeobox 1 (NKX2-1), sodium channel nonvoltage-gated 1 alpha (SCNNA1), surfactant associated protein A1 (SFTPA1), wingless-type MMTV integration site family, member 2 (WNT2), WNT2B, WNT5A, WNT7B, and WNT11. Transcript levels were assessed by quantitative real time polymerase chain reaction (qRT-PCR) [11] using validated primers obtained from Applied Biosystems and normalized to RPL32 (Applied Biosystems, Foster City, CA). Data are presented as means ± the standard error (SE). Group comparisons were considered significantly different $p < 0.05$ as determined by ANOVA and all pairwise comparisons procedure (Holm-Sidak method) (Sigma Plot 11.0 software; Systat Software Inc, San Jose, CA).

FGF2 protein level was assessed from total lung protein homogenate between *Sod3*-/- and *Sod3*+/+ mice as described previously [12]. Total lung homogenate was prepared using 50 mM

Tris-HCL with 2 mM EDTA, pH 7.4 as the lysis buffer (1000 µl) from 4-5 animals/experimental group. Using the Rodent MAP™ version 2.0 of the Rules Based Medicine (Austin, Texas) a panel of markers that included FGF2 was analyzed from the total lung homogenate. Sensitivity level is the least detectable dose (LDD) as provided by Rules Based Medicine. FGF2 measurements were all above LDD. Immunohistochemistry to detect lung FGF2 contrasting *Sod3*$^{-/-}$ and *Sod3*$^{+/+}$ mice was performed [11]. Primary antibody incubation with 10 µg/mL rabbit anti-FGF2 antibody (ab65973, Abcam, Cambridge UK) was performed in 1X PBS + 4% bovine serum albumin (BSA) overnight at 4^{o}C in a humidified chamber. In silico analysis for identification of putative transcription factor binding domains in the promoter region of *Fgf2* (500bp upstream of transcription start site) was analysed using the MatInspector module, Genomatix Software Suite, (version 2.1, Munich, Germany) as described previously [7].

Results

Transcripts encoding HOXA5 and HOXB5, transcription factors that regulate WNT gene expression in the lung [13], increased in the lungs of gene targeted *Sod3*-/- mice compared to control *Sod3*+/+ mice **(Figure. 1).** In turn, WNT2 transcripts increased. The WNT receptors, FZD1, FZD7, and the downstream effector, DVL3, also increased (log 2 fold = 0.8 $\pm$ 0.2p = 0.02, 0.6 $\pm$ 0.1, and 1.0 $\pm$0.2 p = 0.04, respectively) in *Sod3-/-* mice. The previously identified downstream target of canonical WNT/CTNNB1 signaling [14], AXIN2 mRNA increased in *Sod3-/-* compared to control mice (**Figure 1**). In addition, a WNT family member that signals through both the canonical and non-canonical WNT pathway [15, 16], WNT5A increased. Transcripts encoding other WNT signaling proteins (WNT2B, WNT7B, and WNT11), a lung transcriptional factor (NKX2-1), and other lung proteins (ABCA3, SCNNA1, and SFTPA1) were not significantly different between the groups.

The increase in WNT2 transcripts was accompanied by increase in LEF1 (**Figure 2**). LEF1 is a transcription factor and among its targets are FGF genes. FGF2, FGF7, and FGF10

transcripts were increased in *Sod3-/-* compared to control mice (**Figure 2**). Both FGF7 and FGF10 have established roles in embryonic lung development [17, 18]. Because less is currently known about FGF2 in lung development, we further examined FGF2 protein. FGF2 increased in lung homogenate from *Sod3*-/- mice compared to age and sex -matched control mice. Increased immunoreactive FGF2 was detected in the airway epithelium and alveolar parenchyma of *Sod3*-/- mice compared to *Sod3*+/+ mice (**Figure 3**). In silico promoter analysis revealed putative redox sensitive transcription factor binding domains in the promoter region of *Fgf2* that included activating protein 1 (AP1F), MAF and AP1 related factors (AP1R), cAMP responsive element binding protein (CREB), hypoxia inducible factor, bHLH/PAS protein family (HIFF), hepatic nuclear factor 1 (HNF), one cut homeodomain factor (HNF6), histone nuclear factor P (HNFP), nascent polypeptide associated complex and coactivator alpha (NACA), nuclear factor of activated T cells (NFAT), nuclear factor kappa B (NFkB), nuclear receptor subfamily 2 factors (NR2F), nuclear respiratory factor 1 (NRF1) and signal transducer and activator of transcription (STAT) (**Figure 3F).** To obtain a preliminary idea about the expression domains of the increased and decreased transcripts detected in P14 Sod3-/- lungs, we performed a human protein atlas database search (Supplementary figures 1-5). Our findings show expression of immunopositive AXIN2, HOXA5, LEF1, FGF2 and FGF10 in the pneumocytes of normal human lung.

Discussion

Lung development is a complex set of spatial-temporal event that warrants precise coordination among several molecular pathways including homeobox, wingless-type MMTV integration site family, fibroblast growth factors [13, 17, 18, 19]. However, most of our current understanding about these pathways during lung development focuses on early embryonic lung, which entails bud formation, pseudoglandular, cannalicular and saccular stages. In contrast, less is known about alveolarization that starts at late gestational stage (36 weeks) in humans

that continues through childhood and possibly into adolescence [20]. In mice, alveolarization starts at P4 and continues up to P36 [21]. SOD3 activity in the lung is low before birth and increases soon after gestation [22]. Mouse strain (JF1/Msf) with decreased lung volume also exhibited reduction of lung SOD3 levels during the peak phase of alveogenesis compared to C3H/HeJ mice with higher lung volume [7,8].

As a protector of the extracellular matrix, SOD3 binds to and prevents oxidative fragmentation of type I collagen [23], hyaluronan [24], and heparan sulfate proteoglycans (e.g., syndecans) [25], which acts as damage-associated molecular pattern molecules [6, 25, 26]. The extracellular matrix is created by and regulates developmental genes, thus, our interest on SOD3. Changes in SOD3 localization and activity have implications for the neonatal pulmonary response to oxidative stress and the biological activity of NO at birth. In this study we therefore assessed lung developmental genes during peak alveogenesis in mice lacking SOD3.

Mice lacking SOD3 exhibited increased lung HOXA5 and HOX5B transcripts. The *HOX* transcription factor gene family exhibit collinearity, i.e. they exist in linear order within four chromosomal clusters HOXA-HOXD that correspond to the anatomical regions they modulate as well as the timing in which they are activated. In mice, *Hox* genes are expressed mainly in the lung mesoderm, and *Hoxa5* single-mutant and compound *Hoxa5/Hoxb5* double-mutant have abnormal lung branching during embryonic development that results in semi-penetrant neonatal lethality [27, 28]. Defects in gene-targeted mice occur in lung mesenchyme and endodermal-derived epithelium, demonstrating that *Hox* genes can activate secreted proteins that regulate mesodermal-epithelial crosstalk during development.

Among the genes regulated by HOX transcription factors are WNT ligands, which provides spatial information and promotes asymmetric cell division (**Figure 4**). In *Sod3-/-* lung, HOX5A and HOX5B targets, WNT2 and WNT5A transcripts, increased. WNT2 is a secreted ligand that actives the canonical WNT/β-catenin signaling pathway and is predominantly expressed in the

distal lung mesenchyme [29]. In developing human lung, WNT2 transcript is regulated in a spatial-temporal manner with highest levels detected at 17 weeks [19]. Although *Wnt2-/-* deficient mice have normal lung development [29] combined loss of *Wnt2* and *Wnt2b* leads to loss of *Nkx2-1* expression and failure in foregut separation [30]. Gene-targeted *Wnt5a-/-* mice exhibits defective distal lung morphogenesis with truncated trachea and over-expanded distal respiratory airways [31]. Lung specific over expression of WNT5A results in reduced branching and dilated distal airways, and early onset of lung maturation which may affect alveogenesis as well. Increased WNT5A results in increased FGF10 in the mesenchyme and decreased sonic hedgehog in the epithelium thereby affecting the epithelial mesenchymal interaction [15].

LEF1 and AXIN2 transcripts also increased in *Sod3-/-* mouse lung. Activation of the canonical WNT/β-catenin signaling pathway can lead to LEF1 activation [32]. Activated by WNT/β-catenin signaling induction of LEF1 can induce AXIN2 expression [33]. LEF1 and AXIN2 are expressed in a similar manner to WNT2 during human lung development with the highest transcript levels detected for both at gestational week 17 [19]. LEF1, member of the T cell factor transcription factor family is essential for the formation of an active transcription complex with β-catenin and is a nuclear effector of the WNT/β-catenin signaling pathway. The encoded AXIN2 protein is an antagonist of WNT/β-catenin signaling, providing negative feedback to the pathway [34]. Thus, the alteration of these transcripts in *Sod3-/-* mice could have complex integrative interactions.

Lung FGF2, FGF7, and FGF10 transcripts were increased in *Sod3-/-* mice compared to control mice. Of these, the role of FGF7 [18] and FGF10 [15, 17, 18, 35] in lung development have been studied extensively. For example, Yin et al. [14] demonstrated the role of FGF signaling in lung development with inhibition of mesenchymal FGF receptors resulted in decreased mesenchymal WNT2 and LEF1. These findings again support a reciprocal mesothelial/epithelial to mesenchymal loop that controls mesenchymal growth and coordination

of epithelial morphogenesis during pseudoglandular phase of lung development [14]. Impaired epithelial/mesenchymal interaction may affect alveolar septal formation.

Studies in mouse revealed FGF7 as a proliferative factor for lung epithelium and induce type II pneumocyte proliferation [36, 37]. FGF10 is expressed during lung bud formation in splanchnic and distal lung [17] thereby regulating proximal-distal differentiation [38]. The FGF receptors (*Fgfr3,4)* function cooperatively during alveogenesis in murine lung [39]. Powell and colleagues [40] have previously shown the transcript expression of FGFRs in the epithelial cells of large airways (FGFR1,2,4) and alveolar cells (FGFR2,3,4) in rat lungs. The authors had further reported that FGF2 transcript expression decreased with time during post natal lung development in rats [40].

Less is known about the role of FGF2 in lung development. FGF2 is expressed in the lung epithelium, epithelial basement membrane, vascular endothelium, and smooth muscle [41]. Gene-targeted *Fgf2*-/- mice appear normal and without pulmonary defects [42]. However, FGF2 induces proliferation in alveolar epithelial type II cells [43] and airway smooth muscle cells [44,45], and recombinant FGF2 protects from interferon gamma-induced emphysema [46]. In addition, in silico analysis of the FGF2 promoter sequence identified several putative oxidant-mediated transcription factor binding sites (**Figure 3F**). Our results demonstrated increased FGF2 in the airway epithelium and alveolar parenchyma of *Sod3*-/- mice (**Figure 3B-E**) further indicating the plausible role of FGF2 in modulating airway properties of SOD3 deficient mice. Thus, the findings of this study provide promising leads to elucidate the interaction of HOX, WNT, and FGF signaling during alveogenesis in mice lacking SOD3 that warrants further detailed investigations.

To conclude, in this study we identified a potential novel molecular axis involving homeobox/WNT/FGF during alveogenesis in gene targeted *Sod3-/-* mice. The plausible role of FGF2 in lung development is of particular interest. However the study is limited by the lack of

mechanistic data. In future we aim to investigate on the regulation of FGF2 expression by *Wnt2* via *Lef1*. One particular aim would be to study if *Wnt2* treatment or inhibition in vitro is sufficient to alter FGF2 levels in either bronchiolar epithelial cells or type II cells and if *Wnt2* mediated regulation is specific for FGF2 or also influences other FGF members. Moreover, one should consider the fact that both transcript and protein expression studies have been carried out in total lung homogenates whereas lung consists of several cell types (alveolar type I, II; bronchial epithelial cells, endothelial cells, alveolar macrophages etc.). Thus the potential confounding effect of the expression of transcripts from different lung cell population cannot be ruled out. However, in case of FGF2 and SOD3, immunohistochemical analysis demonstrated their expression in bronchial epithelial cells and type II pneumocytes further indicating their plausible interaction. In future from a clinical perspective, it would be interesting to investigate in COPD patients with reduced lung SOD3 levels, the corresponding expression of homeobox, Wnt and FGFs in lung tissue.

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Author Contributions: HS, GDL, and KG conceived and designed the study; TAT, RLB, and KG performed the experiments; TAT, RLB, AM, HS, GDL, KG analyzed and interpreted the data; TAT, HS, GDL, KG wrote the manuscript**.**

Conflict of Interest: None

Figure legends

Figure 1. Lung Homeobox (HOX) and WNT transcripts increase in *Sod3*-/- as compared to strain matched *Sod3*+/+ mice at postnatal day 14. Lung mRNA was isolated, and transcript levels were determined by quantitative RT-PCR (qRT-PCR). Values are mean ± SE (n = 5-6 mice/strain). Statistical significance (*P < 0.05) was determined by ANOVA and by all pairwise comparisons procedure (Holm-Sidak method). **Abbreviation**: **HOXA5**:Homeobox A5, **HOXB5**: Homeobox B5, **WNT2**: wingless-type MMTV integration site family, member 2, **WNT2B**: wingless-type MMTV integration site family, member 2B, **WNT5A:** wingless-type MMTV integration site family, member 5A, **WNT7B**: wingless-type MMTV integration site family, member 7B, **WNT11**: wingless-type MMTV integration site family, member11, **AXIN2**: Axin 2, **SOD3:** Superoxide dismutase 3, extracellular.

Figure 2. Lung lymphoid enhancer binding factor 1 (LEF1) and fibroblast growth factor (FGF) transcripts increase in *Sod3*-/- as compared to strain matched *Sod3*+/+ mice at postnatal day 14. Lung mRNA was isolated, and transcript levels were determined by quantitative RT-PCR (qRT-PCR). Values are mean ± SE (n = 5-6 mice/strain). Statistical significance (*P < 0.05) was determined by ANOVA and by all pairwise comparisons procedure (Holm-Sidak method). **Abbreviation**: **LEF1**: lymphoid enhancer binding factor 1, **FGF2**: fibroblast growth factor 2, **FGF7**: fibroblast growth factor 7, **FGF9:** fibroblast growth factor 9, **FGF10:** fibroblast growth factor 10, **SOD3:** Superoxide dismutase 3, extracellular.

Figure 3. Expression of fibroblast growth factor 2 (FGF2) increased in the lungs of gene targeted *Sod3*-/- lungs compared to control and in silico analysis of *Fgf2* promoter for identification of putative redox sensitive transcription factor binding domains. (**A**) Increased FGF2 protein in the lung homogenates of adult *Sod3-/-* mice (n = 5 female) compared to age- and strain-matched *Sod3*+/+ (C57BL/6J) mice (n=4 female). Values are mean ± SE (n = 4-5 mice/strain). Statistical significance (*P < 0.05) was determined by ANOVA and by all pairwise comparisons procedure (Holm-Sidak method). Immunoreactive FGF2 increased in the airway epithelium of (**C**) *Sod3-/-*mice compared to (**B**) control mice. FGF2 staining also increased in the alveolar parenchyma of **(E)** *Sod3-/-* mice compared to (**D**) control mice. **(F)** Putative redox sensitive transcription factor binding domains in the promoter region of *Fgf2* (500bp upstream of transcription start site).

AP1F: Activating protein 1, **AP1R:** MAF and AP1 related factors, **CREB:** cAMP responsive element binding protein, **HIFF**: hypoxia inducible factor, bHLH/PAS protein family, **HNF**: hepatic

nuclear factor 1, **HNF6:** one cut homeodomain factor, **HNFP:** histone nuclear factor P, **NACA:** nascent polypeptide associated complex and coactivator alpha, **NFAT:** nuclear factor of activated T cells, **NFkB**: nuclear factor kappa B, **NR2F**: nuclear receptor subfamily 2 factors, **NRF1:** nuclear respiratory factor 1, **SOD3:**Superoxide dismutase 3, extracellular. and **STAT:** signal transducer and activator of transcription.

Figure 4. Interactions between HOXA5, WNT2 and fibroblast growth factor signaling. Abbreviation: **HOXA5**: Homeobox A5, **WNT2**: wingless-type MMTV integration site family, member 2, **FZD**: frizzled class receptor, **CTNNB1**: catenin beta 1, **LEF1**: lymphoid enhancer binding factor 1, **FGF2**: fibroblast growth factor 2.

Figure 1

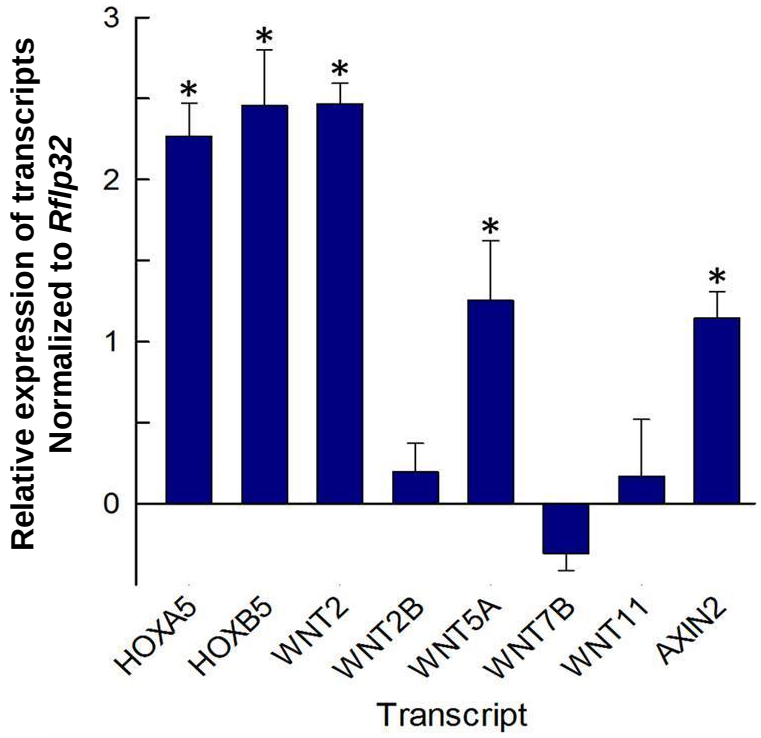


Figure 2

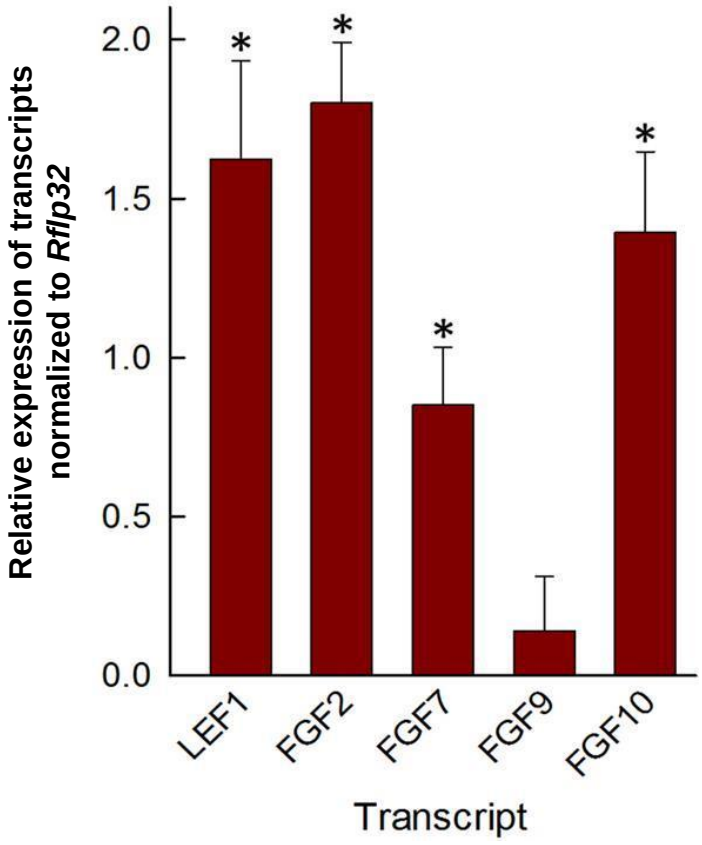
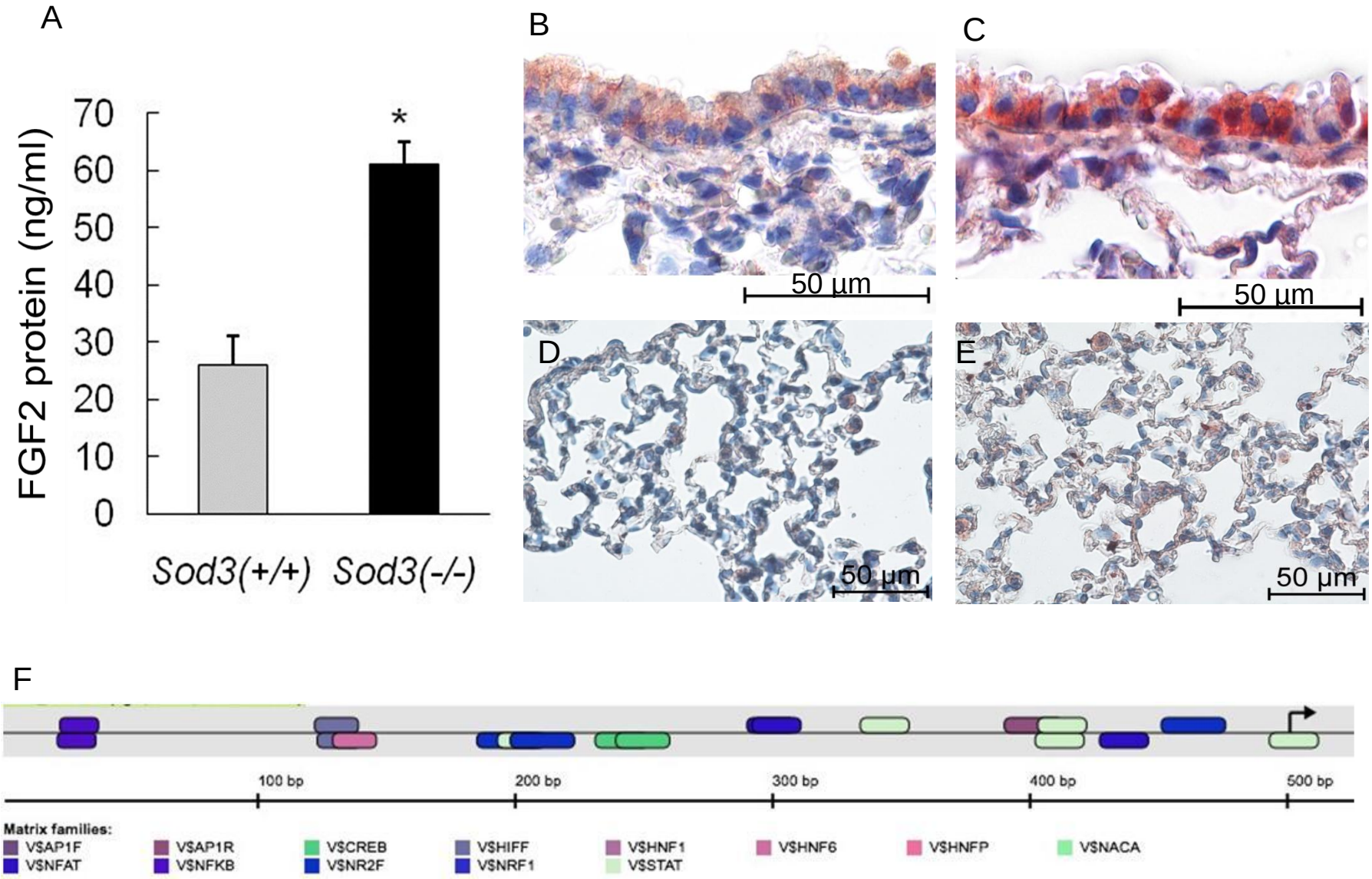


Figure 3


A
FGF2 protein (ng/ml)
70
60
50
40
30
20
10
0
*
Sod3(+/+)
Sod3(-/-)
B
50 µm
C
50 µm
D
50 µm
E
50 µm
F
100 bp
200 bp
300 bp
400 bp
500 bp
Matrix families:
V$AP1F
V$NFAT
V$AP1R
V$NFKB
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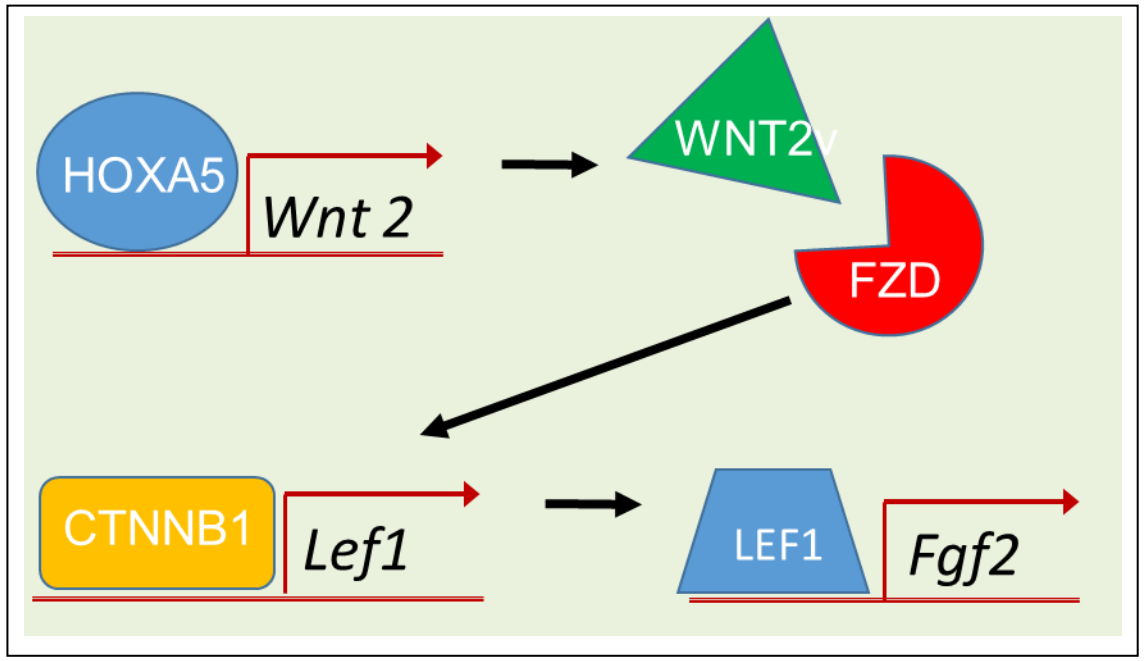
V$STAT
V$HNF6
V$HNFP
V$NACA

Figure 4



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