Roles of Heparan Sulfate in Mesendoderm Differentiation of Human Embryonic Stem Cells

By

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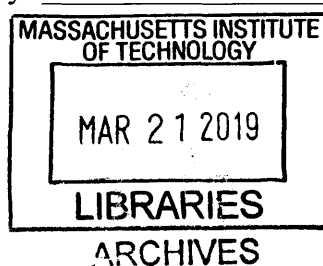
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Abstract
Human embryonic stem cells (hESCs) are remarkable for their ability to self-renew indefinitely and differentiate into any cell type in the human body. The differentiation of hESCs is regulated by intrinsic and extrinsic signals in the stem cell niche. Heparan sulfate proteoglycans (HSPGs) are found on the membrane of all animal cells and have long been implicated in a wide range of cell-cell signaling and cell-matrix interactions. Multiple heparan sulfate (HS)-binding growth factors, such as Wnt, bone morphogenetic proteins (BMP), and fibroblast growth factor (FGF), critically regulate cell fate decisions of ES cells. Here, we showed that HS-deficient derived from hESCs have impaired ability to differentiate into Brachyury-positive mesendoderm (ME) cells. Exogenous addition of heparin partially rescued ME differentiation defect. Furthermore, examination of developmental signaling pathways revealed that HS ablation diminished FGF, Activin A and BMP signaling in differentiated cells. RNA-Seq revealed other biological processes affected by HS deficiency including neurogenesis, bone development and immune responses. Understanding the roles of HS in specific molecular mechanisms that regulate cell fates may provide insights into the complex molecular mechanisms underlying HS-associated human diseases and therefore facilitate the development of therapeutics.

Thesis Supervisor: Laura L. Kiessling
Title: Novartis Professor of Chemistry

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Chapter 1

Human embryonic stem cells

1.1. Overview of human pluripotent stem cells

Human pluripotent stem cells (hPSCs) are well-known for their remarkable ability to self-renew and differentiate into a variety of cell types [1]. hPSCs are powerful tools to study basic developmental processes, regeneration and mechanisms of human genetic diseases [1, 2]. They can be used as models on which to screen new drugs rendering them therapeutic values [1, 2]. They can be manipulated into specific cell fates in vitro serving as an infinite source for some terminally differentiated cells [1, 3]. For example, human cardiac cells have very slow proliferation rate limiting its regenerative capacity after injury [4, 5]. During cardiac infarction, a lot of cardiomyocytes are dead and if dead cardiomyocytes are not replaced by new ones in time, heart functions can be impaired leading to heart failure in the long term [4-6]. hPSCs can be induced to differentiate into functional epicardial progenitor cells and cardiomyocytes in vitro. Those hPSCs derived cardiac cells can be potentially introduced back to injured heart to replace the dead cardiac cells to maintain normal heart functions [4-6].

1.2. Pluripotency

The pluripotency of hPSCs is defined by their ability to differentiate into all derivatives of the three germ layers: ectoderm, endoderm and mesoderm [7]. This unique characteristic distinguishes them from adult stem cells [1]. While hPSCs can produce all cell types in the human body, adult stem cells are multipotent and can become only a limited range of differentiated cell types [1]. A number of molecular markers have been identified to verify the pluripotent status of stem cells [1, 8]. Ideally, hPSCs should be able to proliferate for a year or more in the lab without differentiating [1]. However, the differences between laboratory conditions and endogenous environment in the human body such as oxygen and humidity levels making hPSCs prone to lose their stemness over time [9, 10]. Some well-established pluripotency markers are cell surface proteins SSEA-4 and transcription factors Oct-3/4, Sox2 and Nanog [8, 11]. The ability to verify stem cell pluripotency at the start of an experiment helps

ensure that downstream stem cell proliferation and differentiation studies are conducted on high quality, undifferentiated starting cell populations.

1.3. Differentiation

As mentioned above, hPSCs can give rise to all of the cell types of the three germ layers [7, 12]. Germ layers are formed during an early stage of embryonic development called gastrulation when the inner cell mass that constitutes the blastula begins to differentiate into more-specialized cells [12]. Those more-specialized cells then become layered across the developing embryo and consist of three primary germ layers [12]. The endoderm (inner layer) gives rise to cells in some glands such as the liver and pancreas, the epithelial lining of digestive, reproductive and respiratory systems. The mesoderm (middle layer) gives rise to cells in the skeletal, muscular, circulatory and lymphatic systems. The ectoderm (outer layer) gives rise to cells in the epidermis of skin and its derivatives and the nervous system [13]. Notably, unlike zygotes, hPSCs don't have totipotency because they are unable form extra-embryonic cells (placenta) [12, 13].

1.4. Stem cell niche

The stem cell niche refers to an anatomic location of a tissue in which stem cells reside and remain in a quiescent state. A stem-cell niche provides a specific microenvironment maintaining stem cells in an undifferentiated and self-renewable state [14]. When stem cells are away from their niche, they start to adapt to changing conditions to ultimately stop self-renewing and commence the process of differentiation [14]. The stem cell niche is a dynamic, well-balanced microenvironment which consists of an array of extrinsic signals including signals from the culture medium, nearby cells, and from the extracellular matrix [14-16]. Any disturbance in the niche can affect intercellular and intracellular cues greatly thereby changing downstream genetic circuits. The various biochemical mechanical components within the niche include interactions between stem cells and adhesion molecules, signals from neighboring cells, extracellular matrix components, the oxygen level, growth factors and cytokines, etc [14-17]. Stem cells reside in the stem cell niche, a microenvironment that consists of soluble factors and signals from neighboring cells and the extracellular matrix (ECM) [17, 18]. Despite the significance of the niche, most of the studies to date have been focused on the actuators- the key transcription factors and intracellular signaling pathways that regulate cell fate. Yet the activation of these transcription

factors are usually downstream targets initiated by extrinsic signals and mediated by the "sensors" on cell surface [19, 20]. To fully understand molecular mechanisms that underlie the self-renewal and differentiation of hESCs, it is essential to dissect the processes where extrinsic signals are sensed and "relayed" by the sensors on the cell surface and then integrated into intracellular activities. Studying the stem cell niche is critical not only because we will have a better understanding of how stem cells are controlled in vivo, but we can also apply the knowledge to designing regenerative therapies.

1.5. Differences between human and mouse embryonic stem cells

One commonly asked question is to what extent the findings of studies on the mouse embryonic stem cells are transferable to humans? Both human and mouse embryonic stem cells are considered to be powerful model organisms to study human biology. while they share a lot of similarities, they are quite different in many fundamental aspects [21]. One fundamental difference lies in the ways they are derived during embryonic development [21]. Both human and mouse embryonic stem cells originate from the inner cell mass of pre-implantation blastocysts [21, 22]. However, long period of culture is required for the appearance of human embryonic stem cells allowing explanted cells to progress in vitro to the equivalent of the post implantation mouse embryo, from which mouse epiblast stem cells (EpiSCs) are derived [21, 23, 24]. Therefore, hESCs display a primed state of pluripotency, similar to mouse EpiSCs derived from the post-implantation epiblast [25]. Notably, human and mouse embryonic stem cells are sustained by distinct signaling networks [21]. One instance of the same cell signaling pathway exerting different effects between hESCs and mESCs on cell proliferation and differentiation is FGF signaling pathway. While FGF signaling pathway supports the self-renewal of hESCs, it is dispensable for self-renewal of mESCs [26-29].
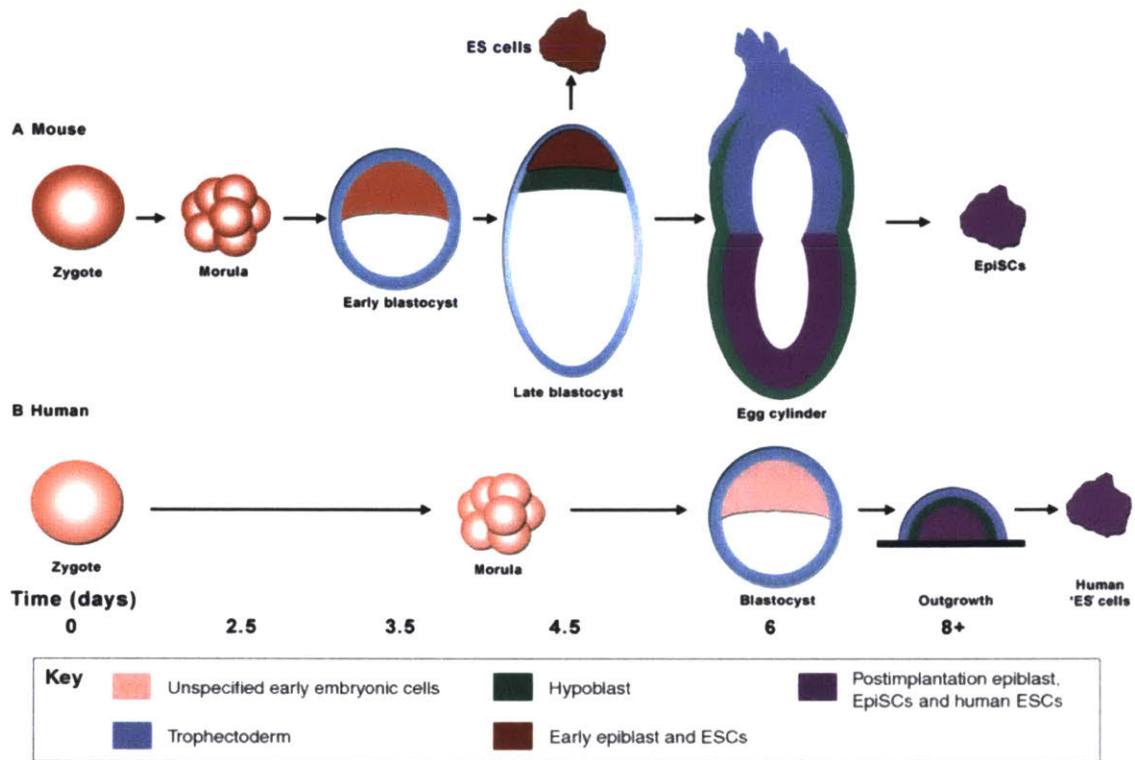
Figure 1. Comparison of derivation of mouse and human embryonic stem cells
(Figure is adapted from figure 3. in [21])

Chapter 2:
Heparan sulfate in pluripotent stem cells

2.1. Heparan sulfate structure, distribution and biosynthesis

Heparan sulfate proteoglycans (HSPGs) are cell surface and extracellular molecules that have long been implicated in a wide range of cell-cell signaling and cell-matrix interactions [19]. HSPGs are composed of a core protein to which long linear glycosaminoglycan (GAG) heparan sulfate (HS) chains are covalently linked [30-32]. HSPGs are classified into three groups based on their location. Syndecans and glycosylphosphatidylinositol-anchored glypicans are found on the cell membrane [33]. HSPGs such as agrin, perlecan and type XVIII collagen are secreted to the extracellular matrix HSPGs [34]. Serglycins are found in secretory vesicles [30].

HS is ubiquitously found at the cell surface and extracellular matrix in all the eumetazoa (animals with true tissues) from Hydra to humans [30]. Whereas heparanosan, a non-sulfated version of heparan sulfate, is found in the bacteria kingdom [30]. HS consists of repeating unbranched anionic disaccharide units of N - acetylglucosamine (GlcNAc) and hexuronic acid residues with sulfation at various positions [35] (Figure 2). HS is structurally heterogenous [31, 32, 36]. Sulfation occurs at different positions of the disaccharide units thereby one HS chain is composed of different proportion of various disaccharides [20, 35]. While the type of hexosamine and the interdisaccharide glycosidic linkage usually remain the same, the abundance, length, epimerization and sulfation patterning of HS are organ/tissue/cell type-specific and change as cells go through differentiation [37, 38].

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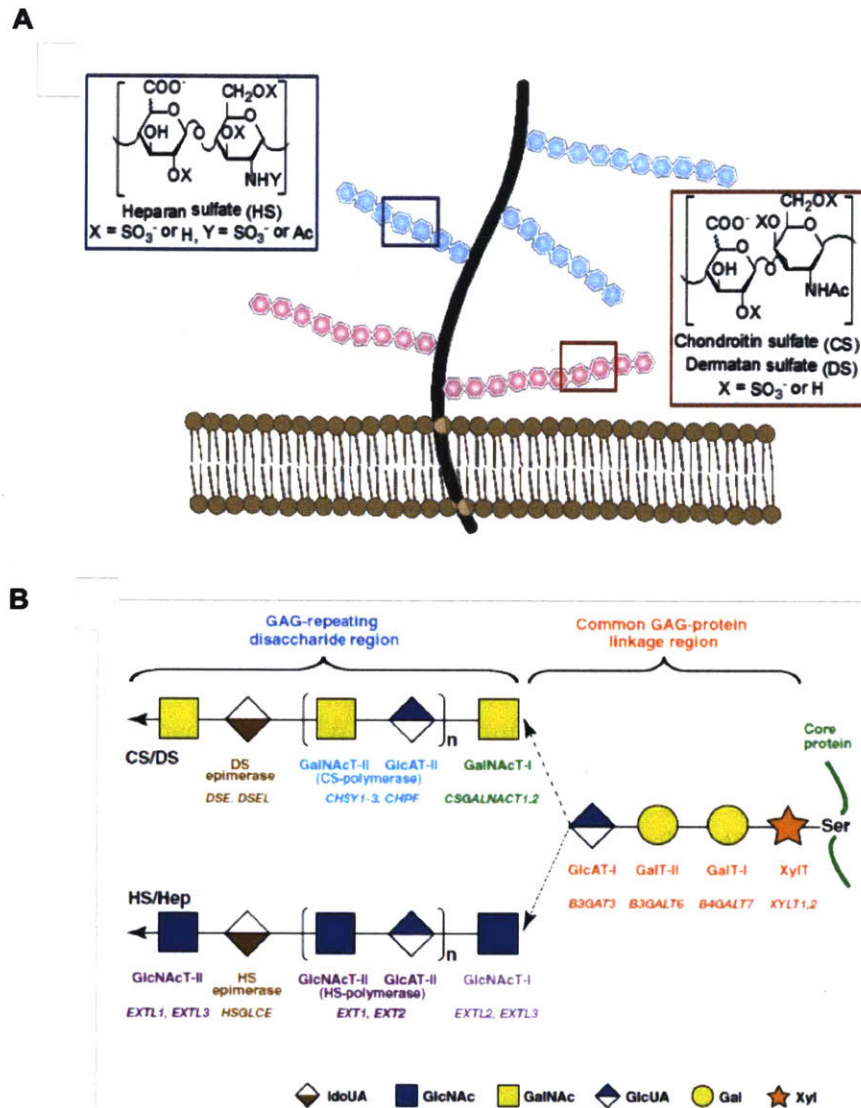
Figure 2. Heparan sulfate proteoglycans (HSPGs) are cell surface and extracellular molecules (A) Proteoglycans have heparan sulfate (HS) and chondroitin sulfate (CS) /dermatan sulfate (DS) GAG chains attached to a core protein. HS is an anionic polysaccharide composed of repeating disaccharide units of N-acetylglucosamine (GlcNAc) and either glucuronic acid (GlcA) or iduronic acid (IdoA) with negatively charged sulfate groups (SO_3^-) throughout the chain. (B) Biosynthetic assembly of the GAG backbones by various glycosyltransferases. Both CS and HS have this common linkage region, but the composition of repeating region is different. *EXT1* encodes for a glycosyltransferase that is responsible for the chain polymerization of HS/heparin. (Figure B is adapted from figure 1 in [39])

The biosynthesis and assembly of HS linkage tetrasaccharide region to the core protein start in the endoplasmic reticulum with the transfer of xylose (Xyl) to specific serine residues (Ser) within the core protein followed by the attachment of two Gal residues and one GlcA [40, 41]. The polymerization of HS chains is initiated by adding a GlcNAc residue to the linkage region catalyzed by exostosin-like-2/3 (EXTL2/3) [42]. The HS chain is then elongated by the alternating addition of GlcA and GlcNAc residues catalyzed by polymerases (EXT1/EXT2 heterodimer complex) in the Golgi apparatus [42-44]. As chains elongate, they undergo extensive modifications including N-deacetylation/N-sulfation catalyzed by N-deacetylase/N-sulfotransferase (Ndst), epimerization by glucuronyl C5-epimerase (Hsepi) and O-sulfation by O- sulfotransferases (Hs2st, Hs6st, Hs3st) [36, 45].

The vast structural diversity endows HS with the ability to interact with a wide spectrum of proteins, such as growth factors, chemokines, morphogens, extracellular matrix components, enzymes, etc. (summarized in Table 1)[38, 46, 47]. Therefore, HS has been implicated in a variety of processes from embryonic development, homeostasis, to human diseases [46]. In the extracellular matrix [47], HS participates in establishing and maintaining morphogen gradients that play central roles in establishing the position and identity of cells to create the architecture of developing tissues [38, 46, 47]. HS can bind cytokines to control their localization and alter their activity [38, 48]. It is well-known that HS binds growth factors and function as a co-receptor on the cell surface thereby enhancing the formation of the ligand-receptor complex [38, 46, 47].

Table 1. Heparan sulfate binding proteins (adapted from TABLE II in [38])

TABLE II
Heparan sulfate binding proteins*.

Cell surface	References
L-selectin and P-selectin	(Ma and Geng 2000)
N-CAM (Neural Cell Adhesion Molecule)	(Cole et al. 1986)
PECAM-1 (Platelet Endothelial Cell Adhesion Molecule)	(Watt et al. 1993)
FGF receptor	(Powell et al. 2004)
HIP (Heparin/Heparan Sulfate Interaction Protein)	(Rohde et al. 1998)
MAC-1 (Monocyte Adhesion Molecule)	(Coombe et al. 1994)
Extracellular matrix	**References**
Collagens	(Sasisekharan et al. 2002)
Fibronectin	(Capila and Linhardt 2002)
HB-GAM (Heparin Binding Growth Associated Molecule)	(Taylor and Gallo 2006)
Laminin	(Utani et al. 2001)
Tenascin	(Saito et al. 2007)
Thrombospondin I and II	(Nunes et al. 2008)
Vitronectin	(Wilkins-Port and McKeown-Longo 1996)
Growth factors	**References**
HB-EGF family (Heparin Binding – Epidermal Growth Factors)	(Aviezer and Yayon 1994)
FGF family (Fibroblast Growth Factors)	(Gambarini et al. 1993)
VEGF (Vascular Endothelial Growth Factor)	(Iozzo and San Antonio 2001)
HDGF (Hepatoma Derived Growth Factor)	(Dietz et al. 2002)
PlGF (Placenta Growth Factor)	(Athanassiades and Lala 1998)
PDGF (Platelet-Derived Growth Factor)	(Sasisekharan et al. 2002)
TGF-β (Transforming Growth Factor-β)	(Sasisekharan et al. 2002)
HGF (Hepatocyte Growth Factor)	(Derksen et al. 2002)
Cytokines/Chemokines/Morphogens	**References**
BMP (bone morphogenetic protein)	(Hacker et al. 2005)
IL-1, -2, -3, -4, -5, -7, -8, -10, -12 (Interleukin)	(Koopmann et al. 1999)
IP-10 (Interferon -γ inducible protein 10)	(Handel et al. 2005)
CCL-2 (CC-chemokine ligand)	(Johnson et al. 2005)
GM-CSF (Granylocyte Macrophage Colony Stimulating Factor)	(Raman et al. 2005)
MCP-1, MCP-4 (Monocyte Chemoatractant Protein)	(Johnson et al. 2005)
RANTES (Regulated on Activation Normal T cell Expressed and Secreted)	(Johnson et al. 2005)
TNF-α (Tumor Necrosis Factor)	(Handel et al. 2005)
MIP-1 (Macrophage Inflammatory Protein)	(Vlodavsky et al. 2002)
PF-4 (Platelet factor 4)	(Sulpice et al. 2002)
Hh (Sonic Hedgehog)	(Hacker et al. 2005)
Wnt (Wingless wg)	(Hacker et al. 2005)
Others	**References**
DNA and RNA polymerases	(Furukawa and Bhavanandan 1983)
Superoxide dismutase	(Nozik-Grayck et al. 2005)
Angiogenin	(Soncin et al. 1997)
Cathepsins B and G	(Almeida et al. 2001)
Neutrophil elastase	(Campbell and Owen 2007)
Annexin V	(Mulloy and Linhardt 2001)
Prion	(Ben-Zaken et al. 2003)
β-amyloid protein	(Patey et al. 2008)
Na$^+$/Ca^{2+} exchanger protein	(Shinjo et al. 2002)
Myosin ATPase	(Tersariol et al. 1992)

2.2. Heparan sulfate-associated human diseases

Hereditary multiple exostoses (HME) also called multiple osteochondroma (MO) is an autosomal dominant hereditary disorder linked to germline heterozygous loss-of-function mutations in *EXT1* or *EXT2* that encode glycosyltransferases responsible for HS chain elongation [44, 49-51]. One in 50,000 children is affected by HME with almost 100% penetration [52], which means the effect of HS defect occurs regardless of the environmental factors present. Mutations in *EXT1* or *EXT2* lead to the premature termination and degradation of EXT protein which does not function normally, resulting in a systemic HS deficiency [53]. The HS levels observed in surgical retrieval specimens of human exostosis cartilage were found to be very low and apparently lower than the 50% levels presumably caused by a single heterozygous EXT mutation [54, 55]. These low levels can be explained by the fact that the enzymes EXT1 and EXT2 form a complex that acts to form the HS polysaccharide core. The most evident trait of HME is the growth of benign cartilaginous-bony outgrowths around areas of active bone growth [54, 56]. Some patients also display other issues such as delayed wound healing, learning disabilities, autism-like social problems and impaired pancreatic insulin secretion [57-60], indicating that HS regulates a broad array of physiologic processes. Exostosis formation can cause shortened stature and skeletal deformities and osteoarthritis over time, with transformation of benign exostoses to malignant chondrosarcomas in 2-5% of patients [59, 61-63]. Current treatments include surgery, physical therapy and pain management [59, 62]. Since the exostoses exhibit an intriguing growth plate-like structure, it is hypothesized that HS deficiency causes ectopic chondrogenesis from chondrocytes in the growth plate and perichondrial/groove cells in the borderline growth plate [64, 65]. In brief, the pathologic mechanisms of HME remain unclear and many studies have been carried out in mice and human somatic cells in order to solve this question.

2.3. Heparan sulfate associated knockout mouse studies

Numerous mouse models have been developed to study the functions of HS in development. *Ext2*-null mice fail to undergo gastrulation, lack organized mesoderm and extraembryonic tissues, and die during early development, illustrating the importance of HS in early mammalian embryogenesis [66]. *Ext1*- null mice were also found to be embryonic lethal [67]. Conditional

ablation of *Ext1* in mice leads to dysregulation of BMP signaling and severe skeletal defects [68]. Heterozygous null *Ext1*^{+/-} mice don't show any obvious signs of HME and lack any evident skeletal phenotype [69]. Heterozygous null *Ext2*^{+/-} mice showed minor alternations in growth plate similar to those in heterozygous null *Ext1*^{+/-} mice. However, about 20% of the *Ext2*^{+/-} mice formed one or more ectopic bone growths (exostoses) in ribs, supporting the idea that partial loss of HS results in exostosis formation [66]. Double heterozygous *Ext1*^{+/-}: *Ext2*^{+/-} mice showed more obvious skeletal defects such as bowed forearms and nearly half of the mice developed stereotypic exostoses with growth plate-like characteristics [70].

2.4. Research Goal and Objectives

As discussed in Chapter 1 and 2, the pluripotency of hESCs is tightly regulated by signals in the stem cell niche. As a critical component of the stem cell niche, HS have been shown to interact with a variety of proteins. Although multiple mouse models have been generated to study the significance of HS during development, studies investigating the effects of HS at the human stem cell level are still lacking. Filling this gap will not only complement pre-existing mouse studies, but, more importantly, will provide better insights into the pathologic mechanisms linking HS deficiency and human diseases. The goal of this study is to fill this gap by deciphering the roles of HS in the differentiation of hESCs, with a focus on mesoderm and endoderm differentiation.

In achieving this goal, I aim to address three main research objectives:
Objective 1: To examine the mesendodermal and endodermal differentiation potential of HS-deficient cells; Objective 2: To uncover the impact of HS deficiency on developmental signaling pathways during differentiation; Objective 3: To uncover new biological processes and pathways associated with HS deficiency by transcriptome profiling.

Chapter 3:
Heparan sulfate deficiency and mesendoderm differentiation

3.1. Background

Previous studies in our lab identified that chemically defined peptide-presenting surfaces can maintain long-term self-renewal of hESCs [3]. The most intriguing finding was that surfaces displaying a heparin-binding peptide (GKKQRFRHRNRKG27 derived from vitronectin) were most effective at supporting cell adhesion and self-renewal [3, 71, 72]. Analysis of the adhesion mechanism indicated that hESCs interacted with the surfaces through their cell-surface glycosaminoglycans (GAGs) [3]. Given that HSPGs can mediate many developmental signaling pathways in mammalian cells, we focus our attention on heparan sulfate and predict it is a key player in the self-renewal and cell fate decisions of hESCs [46].

3.2. Generation of human $EXT1^{-/-}$ cells

To determine the functional roles of HS in hESCs, Dr. Masuko and Dr. Zaltsman in the lab generated and characterized a HS-deficient cell line from H9 cell line by CRISPR/Cas9-mediated targeting of gene $EXT1$, which encodes for a glycosyltransferase that polymerizes the HS backbone chain [44, 73]. To isolate HS-deficient cells from HS-containing ones, cells were plated on a synthetic surface that engages cells surface GAG chains [3, 71, 72]. Since HS-containing cells would adhere to the GAG-binding surface but HS-deficient ones would not, non-adherent cells were then replated on Matrigel-coated surfaces on which they could employ other adhesion receptors for attachment. After a series of enrichment steps, HS-deficient cells were purified and then derived into single colonies. Sanger sequencing revealed that both alleles incorporated frameshift mutations at the site targeted by gRNA. HS binding assay was carried out by flow cytometry and immunostaining to determine whether HS expression is completely abolished in $EXT1^{-/-}$ cells. Mass spectrometry analysis was also employed to assess the presence of heparan sulfate, chondroitin sulfate and hyaluronic acid. Whereas HS was detected in WT cells, no HS was detected $EXT1^{-/-}$ cells by all three analyses. These results indicated that $EXT1^{-/-}$ cells are indeed HS-deficient.

3.3. Mesendoderm differentiation of WT and $EXT1^{-/-}$ cells

Some preliminary work done in the lab showed that $EXT1^{-/-}$ cells failed to express mesodermal and endodermal markers upon spontaneous differentiation by Embryoid Body (EB) formation test. In addition, direct cardiomyocyte differentiation experiments showed that while WT cells were induced to become mature, beating cardiomyocytes, $EXT1^{-/-}$ cells were unable to become functional cardiomyocytes. Since mesoderm and endoderm share a progenitor stage named mesendoderm (ME), we hypothesized that $EXT1^{-/-}$ cells are unable to differentiate into mesendoderm. To evaluate the differentiative capacity of $EXT1^{-/-}$ cells, I first induced them to mesendoderm using a chemically-defined CHIR protocol [74]. CHIR, a small membrane-permeable molecule, is a potent and highly selective inhibitor of glycogen synthase kinase 3 (GSK-3). Since GSK-3 targets intracellular β-catenin for degradation, inhibiting GSK-3 with CHIR leads to the activation of canonical Wnt/β-catenin signaling, which in turn initiates mesendoderm (ME) differentiation [75]. The aim of applying this chemically-defined CHIR protocol is to avoid any introduction of exogeneous HS into the culture medium, which may partially or completely mask the effects of HS deficiency. In details, cells were seeded at 500,000 cells per well in a 6-well non-TC plate at day -3 and cultured in mTESR-E8 media on Vitronectin XF™ for three days until reaching 100% confluency. To induce direct ME differentiation, cells were treated with 6 μM CHIR in RPMI for 24 hours before being collected for analysis (Figure 3E). At day1, almost all WT cells were Brachyury-positive, however, only around 25% of $EXT1^{-/-}$ cells expressed Brachyury (Figure 3D). Brachyury and MIXL1 are both transcription factors. Brachyury has a DNA binding activity in the T-domain and is commonly used as a hallmark for mesendoderm. MIXL1 is required for proper axial mesendoderm morphogenesis and endoderm formation. A similar difference in T (the gene name for Brachyury) and $MIXL1$ expression was also observed at transcriptional level by qPCR (Figure 3B). Moreover, Western blot and immunostaining for Brachyury confirmed indeed $EXT1^{-/-}$ cells expressed less Brachyury at the protein level (Figure 3A, 3C), indicating $EXT1^{-/-}$ cells have impaired ability to differentiate into mesendoderm. Interestingly, a remarkable heterogeneity of Brachyury expression was observed within the same population of $EXT1^{-/-}$ cells, which may be caused by (1) the culture conditions wasn't completely HS-free, a small amount of HS still remained in the system. (2) cells exist in a dynamic, metastable state. In other words, cells are heterogeneous by nature with respect to endogenous cell signaling activity, thus having different differentiation potentialities [76, 77].
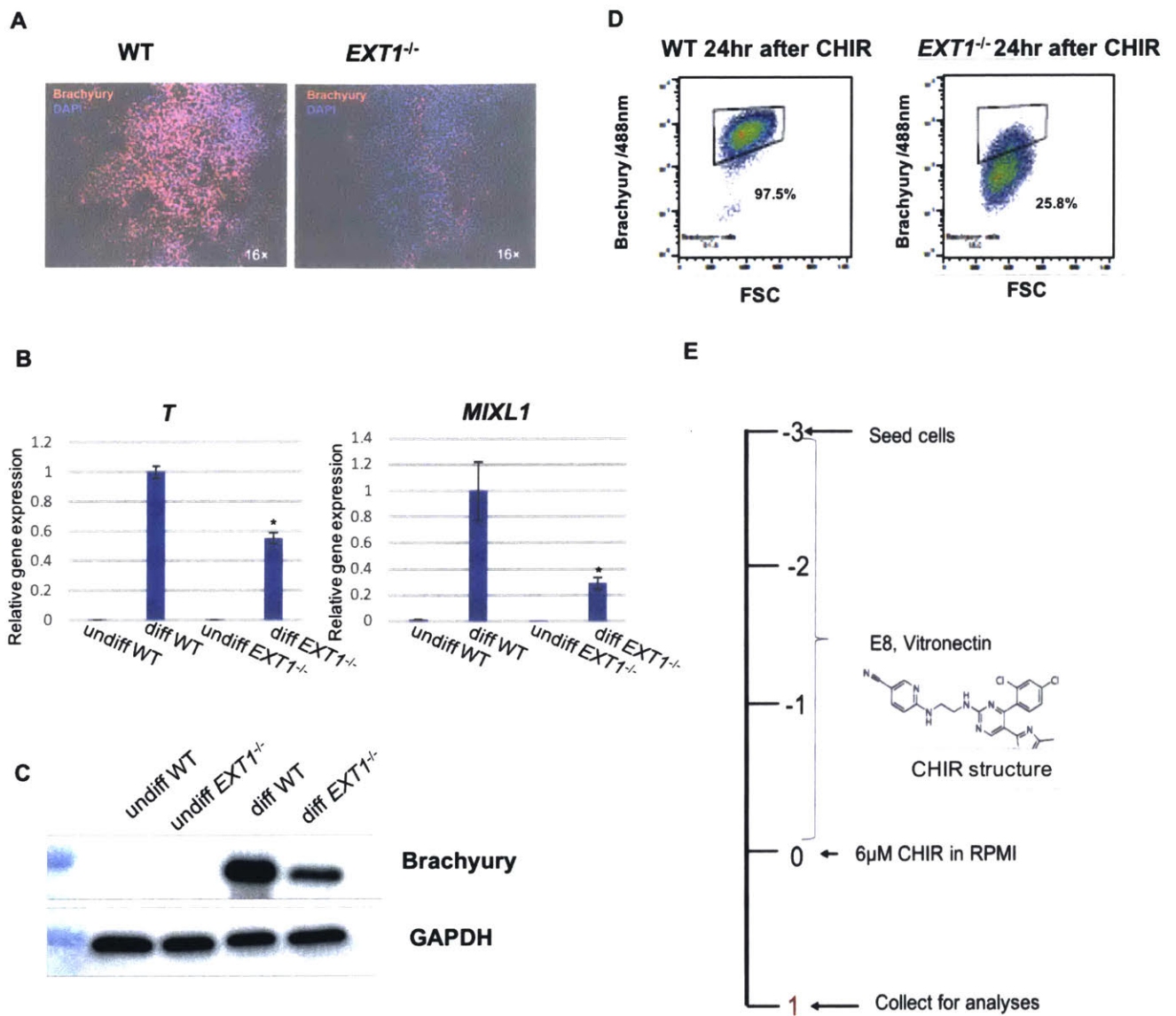
Figure 3. HS-deficient cells have impaired ability to differentiate into Brachyury-positive cells (A) Immunostainings of WT and *EXT1*^{-/-} cells stained for Brachyury and counterstained for DAPI upon mesendoderm differentiation. (B) qPCR analysis of ME markers *Brachyury* and *Mixl1* in WT and *EXT1*^{-/-} cells. (C) Protein level of Brachyury and GAPDH as a loading control. (D) Flow cytometry analysis of Brachyury in differentiated WT and *EXT1*^{-/-} cells. The black box represents a gate for Brachyury-positive events. A total of 100,000 individual events were collected. (E) Chemically-defined mesendoderm differentiation protocol. 6 μM CHIR in RPMI was used to induce differentiation on day 0.
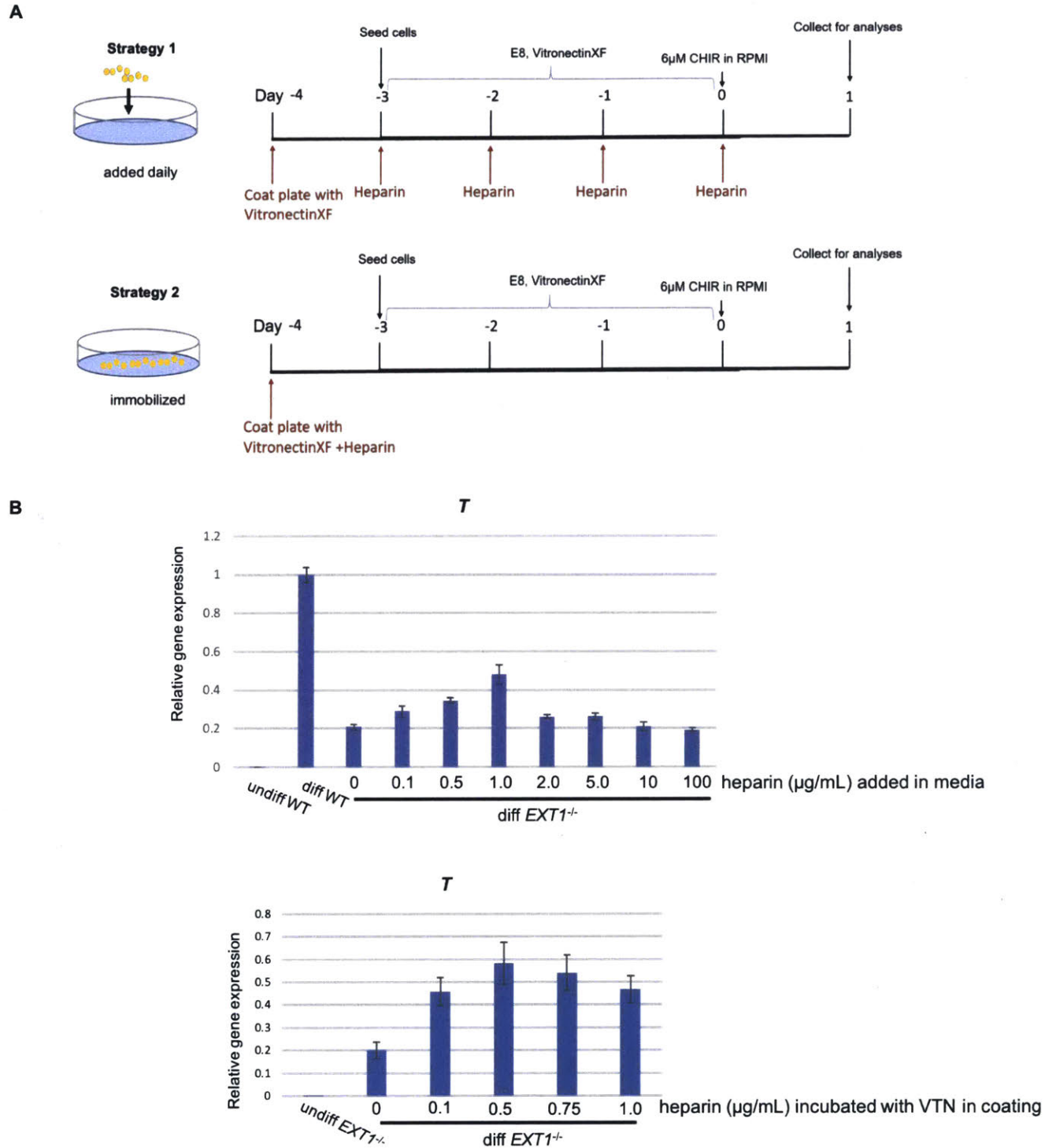
3.4. Heparin complementation strategy

Next, to validate that HS deficiency is indeed the cause for ME defect, *EXT1*$^{-/-}$ cells were supplemented with varying concentrations of heparin during ME differentiation. Heparin is a structural analog of HS and has been used as a substitute for HS in mouse studies [35]. In mESCs, the addition of heparin was able to rescue the defective phenotypes resulting from HS deficiency or downregulation [29, 78, 79]. Thus, we hypothesized that the addition of soluble heparin to the differentiation medium can restore the ME differentiation in *EXT1*$^{-/-}$ cells.

I designed and carried out two heparin complementation strategies. In strategy 1, various concentrations of heparin were added to the culture and ME-inducing media on a daily basis until cells were collected for analysis (Figure 4A). Brachyury expression was evaluated at transcript level by qPCR and at protein level by Western blot. Overall, Brachyury expression was partially rescued in *EXT1*$^{-/-}$ cells with the largest rescue effect observed at 1 μg/mL heparin (Figure 4B, 5). Moreover, a positive correlation was observed between Brachyury expression and heparin concentrations from 0.1 to1 μg/mL, but Brachyury expression actually decreased a little bit as heparin concentration went from 1 μg/mL to 100 μg/mL (Figure 4B). One explanation for this increase/decrease effect in heparin rescue is multivalent ligand-receptor binding [80] (illustrated in Figure 6). For instance, BMP signaling is crucial for ME development and it is known that one BMP ligand needs to form a ternary structure with two BMP receptors (BMPR) on the cell surface in order to activate downstream intracellular components [88-90]. Under normal conditions, HS facilitates the binding of one BMP ligand to two BMPRs which in turn triggers downstream BMP signaling [88-90]. However, if too much HS/heparin is present, the binding of BMP ligand to BMPRs changes from 1:2 to 1:1, which in turn shuts down the downstream signal transduction, leading to lower T expression. Additionally, I modified this strategy to see whether a daily dose of heparin to the culture media is actually dispensable for the rescue effect. In this modified version of strategy 1, *EXT1*$^{-/-}$ cells were supplemented with various concentrations of heparin only on the day of ME differentiation induction (day 0) (Figure 7A). No significant rescue effect was observed for any of the heparin concentrations tested. This

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result suggested that priming $EXT1^{-/-}$ cells with heparin beforehand is indispensable for the effective rescue of ME differentiation (Figure 7B).

In strategy 2, surfaces were coated with vitronectin and varying concentrations of heparin before cell seeding with no additional heparin added to the culture medium during the entire process (Figure 4A). We assumed that the incubation of heparin with vitronectin substrate would result in the binding of the two components due to charge interaction and the binding of heparin to a GAG binding motif in vitronectins [3]. A rescue in T expression was observed at all five heparin concentrations tested ranging from 0.1 µg/mL to 1µg/mL (Figure 4B). A similar trend in T expression in correlation to increasing heparin concentrations was also observed here, probably an outcome of multivalent ligand-receptor binding (Figure 6). Notably, this strategy rescued ME differentiation at a higher efficiency as T expression peaked at 0.5 µg/mL as opposed to 1 µg/mL of heparin in strategy 1 (Figure 4B). Given cells adhered to vitronectin throughout the entire process, heparin bound to a substrate would likely contact and interact with cells more effectively to induce signal transduction than heparin added directly to the media.

Figure 4. ME differentiation defect can be partially restored in the *EXT1*^{-/-} cells by chemical complementation strategy

(A) Schematic diagrams of the two chemical complementation strategies used. In strategy 1, *EXT1*^{-/-} cells were supplemented with 0-100 μg/mL of heparin on a daily basis. In strategy 2,

plates were pre-coated with vitronectin and 0-1.0 μg/mL of heparin before cell seeding. (B) qPCR analysis of *T* expression in cells collected 1 day following ME differentiation induction.
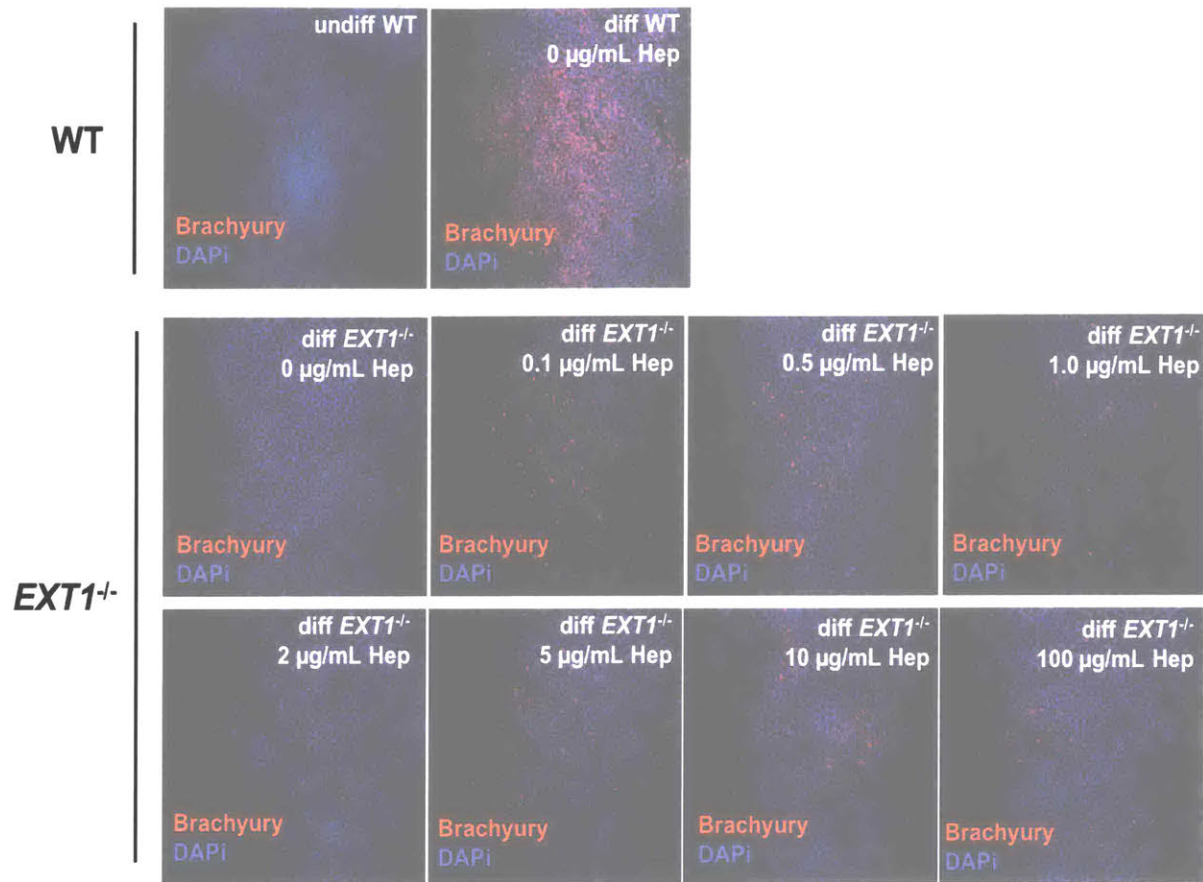
Figure 5. Complementation of ME differentiation with 0-100 μg/mL heparin

Immunostainings of WT and *EXT1*$^{-/-}$ cells stained for Brachyury (red) and counterstained with DAPI (blue) following one day of ME differentiation. *EXT1*$^{-/-}$ cells were supplemented with 0-100 μg/mL soluble heparin on a daily basis before staining.
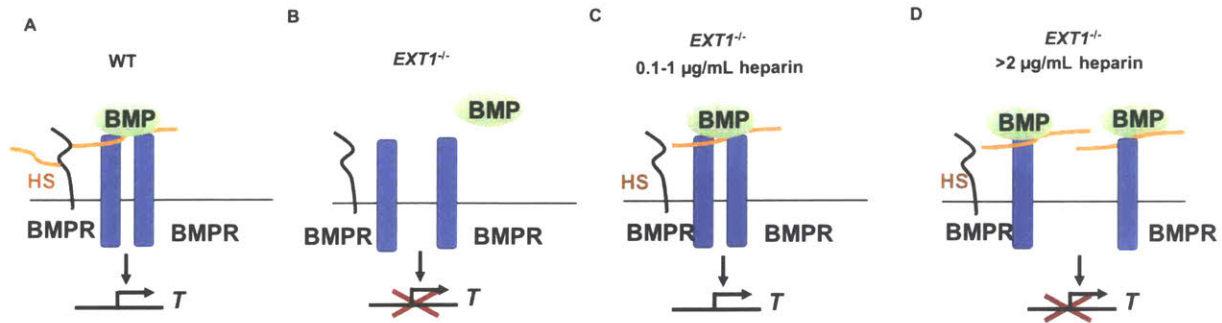
Figure 6. Proposed mechanisms for partial rescue of ME differentiation by heparin in *EXT1*$^{-/-}$ cells: for many cell surface receptors, Multivalent ligand-receptor binding is essential for signal transduction [80]. For example, BMP signaling is essential in ME differentiation, and one BMP ligand needs to bind to two BMP receptors (BMPR) simultaneously in order to activate downstream signal transduction [81, 82].

(A) In WT cells, HS facilitates the binding of one BMP ligand to two BMPRs on the cell surface, activating *T* expression. (B) In *EXT1*$^{-/-}$ cells, BMP-BMPRs interactions is weakened by the lack of HS on the cell surface, resulting in inactivated *T* expression. (C) 0.1-1 µg/mL heparin can restore the binding of one BMP to two BMPRs in *EXT1*$^{-/-}$ cells, leading to reactivated *T* expression. (D) When too much heparin is present in the media, the binding between BMP and BMPR changes from one-to-two interaction to one-to-one interaction. This one-to-one interaction cannot activate downstream signal transduction, resulting in lower *T* expression.
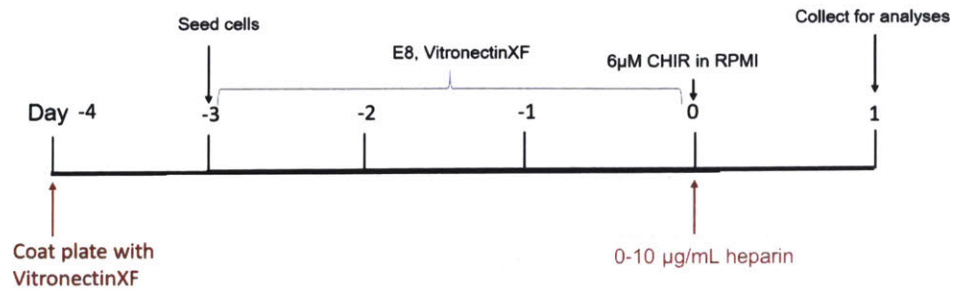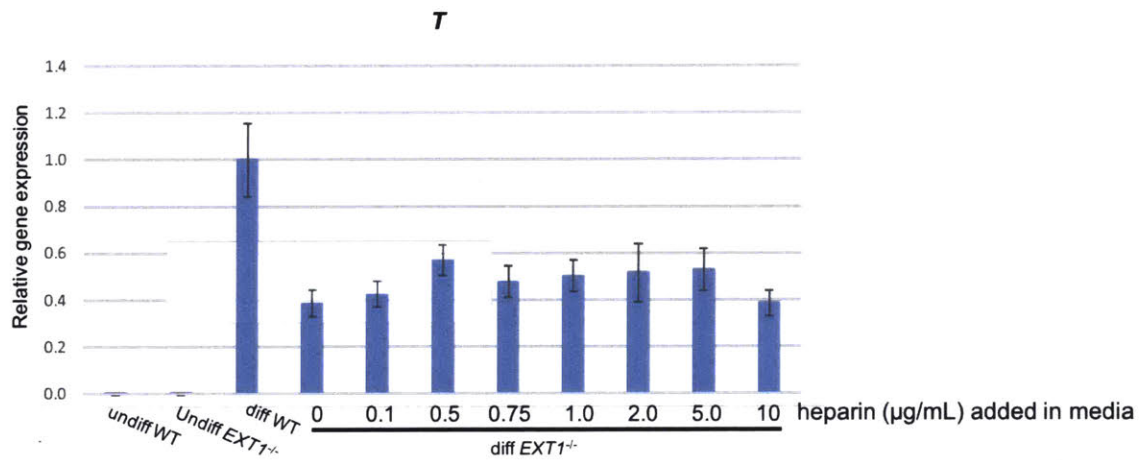
Figure 7. ME differentiation defect cannot be rescued in the *EXT1⁻/⁻* cells by addition of heparin at day 0 of differentiation

(A) Schematic diagram showing the chemical complementation strategy used. *EXT1⁻/⁻* cells were supplemented with 6 μM CHIR and 0-10 μg/mL of heparin on day 0. (B) qPCR analysis of *T* expression in cells collected 1 day upon ME differentiation.

3.5. Brachyury reporter cell line

In aid in deciphering the functional roles of HS in ME differentiation, I set out to generate a HS-deficient Brachyury reporter human cell line by CRIPSR/Cas9-mediated targeting of *EXT1* in an existing Brachyury reporter human embryonic stem cell line- H9-T-2A-EGFP-PGK-Puro (hereafter referred to as WT Brachyury reporter cells) (Figure 8A) [83]. Following CRISPR/Cas9-mediated editing, D5 clone was isolated and cell-surface HS levels were assessed by flow cytometry prior to sanger sequencing (Figure 8B). Sanger sequencing identified

frameshift mutations on both alleles of *EXT1* in D5 clone (hereafter referred to as *EXT1*^{-/-} Brachyury reporter cells) (Figure 9).

To complement prior results, I induced direct ME differentiation in WT and *EXT1*^{-/-} Brachyury reporter cells. I noticed immediately that WT and *EXT1*^{-/-} Brachyury reporter cells were more sensitive to 6 µM CHIR treatment, so I lowered CHIR concentration to 4 µM and tried ME differentiation under both E8/Vitronectin and mTeSR/Matrigel conditions. Under chemically-defined conditions (E8/Vitronectin), treatment with 4 µM CHIR activated Brachyury promoter activity in WT Brachyury reporter cell line but not in *EXT1*^{-/-} Brachyury reporter cell (Figure 10A). In contrast, treating cells cultured under chemically-undefined conditions (mTeSR/Matrigel) with 4 µM CHIR activated Brachyury promoter activity in both WT and *EXT1*^{-/-} Brachyury reporter cell lines (Figure 10B), suggesting the amount of HS existed in mTeSR and Matrigel was enough to rescue the ME defect of *EXT1*^{-/-} Brachyury reporter cells.
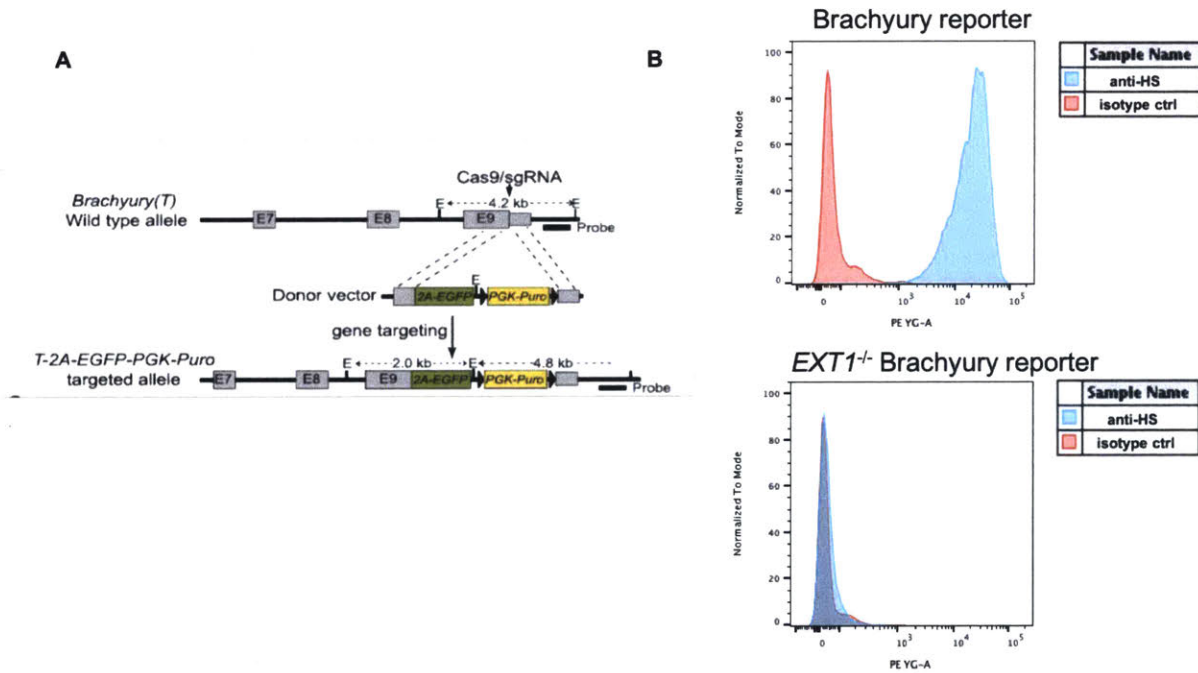
Figure 8. Construction of *T* reporter human embryonic stem cell line and validation of *EXT1*^{-/-} Brachyury reporter cell line (A) Gene targeting strategy of knock-in T-2A-EGFP-PGK-Puro cassette to replace the endogenous T stop codon, facilitated by the CRISPR/Cas9. Gray boxes indicate the exons of the endogenous gene. The arrow indicates the position of the Cas9/sgRNA

cut site. (Figure adapted from [83]) (B) Flow Cytometry analysis of cell surface expression of HS in Brachyury reporter and *EXT1*⁻/⁻ Brachyury reporter cells.
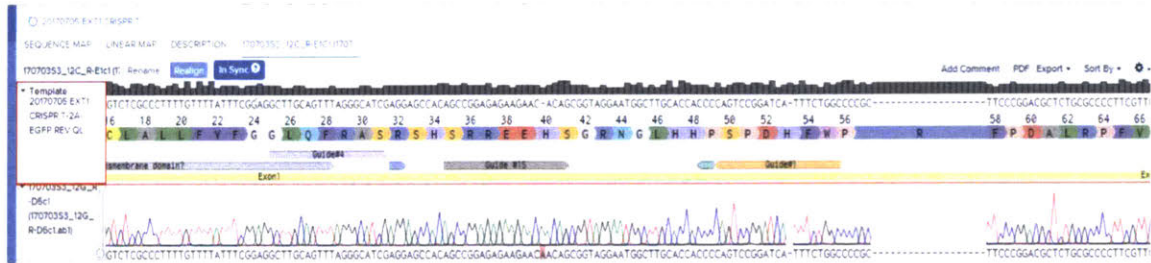
Figure 9. Determining mutations at PAM site of *EXT1* in CRISPR/Cas9 treated D5 clone by mono-allelic sequencing. In order to confirm that both alleles were effectively modified. the genomic region in which the indels were expected to be was amplified, cloned into a TOPO vector. 4 TOPO clones from D5 were sent to sequencing. Same frameshift mutation ("A" in red box) in *EXT1* gene was obtained for all 4 TOPO clones.
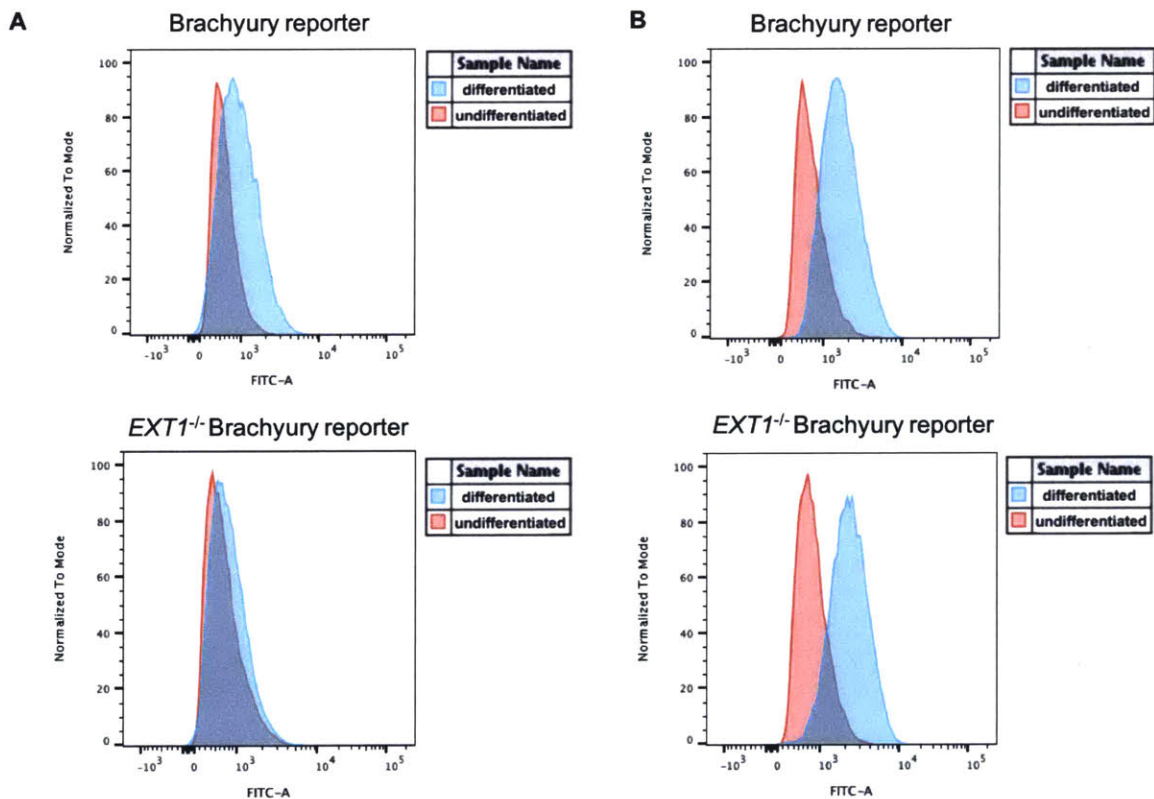
Figure 10. Histograms of FITC measured by flow cytometry in Brachyury reporter cells using chemically-defined (A) and -undefined (B) ME differentiation protocol. Cells were collected following one day of differentiation, rinsed with PBS and stained with live-dead stain. FITC (X-axis) indicates Brachyury expression.

3.6. Summary

Findings described in this section highlight the indispensable role of HS in ME differentiation under chemically-defined conditions. Additionally, exogenous heparin can partially restore the ME differentiation in *EXT1*$^{-/-}$ cells. However, some concerns have arisen regarding the chemical complementation experiments: (1) While HS is structurally related to heparin, it is a shorter, much more sulfated HS variant that is restricted to mast cells [35]. These differences in structure and localization give them slightly different functions. For instance, heparin functions as an anticoagulant whereas the anticoagulant activity of HS is much lower [35]. Thus, an experiment worth doing is to repeat the rescue experiments with HS to see if we can still observe similar results. (2) Although HS exists in the ECM, it is mostly found in the glycocalyx coating cell surface [30]. One concern that has been raised about using exogenous heparin to rescue HS deficiency is how close this rescue method mimics naturally occurring process where heparin-growth factor complexes recruit and cluster receptors on the cell surface. One way to solve this issue is by tethering heparin to the cell surface proteins through a HaloTag protein or other techniques. It's likely that this stable, long-term HS presentation on the cell surface would rescue ME differentiation in a more efficiently way.

Chapter 4:

Molecular mechanisms of mesendoderm differentiation defect in *EXT1*$^{-/-}$ cells

4.1. FGF signaling

Fibroblast growth factor (FGF) signaling is crucial for the survival, migration and differentiation of hPSCs [84]. FGF signaling pathway is initiated by the binding of one FGF ligand to two FGF receptors (FGFR) [85]. This ligand-receptor interaction results in the autophosphorylation of tyrosine residues in the intracellular domain of an FGFR, which triggers downstream signaling cascades [85]. It is well-established that HS controls the bioavailability of secreted FGF in the ECM, and on the cell surface, HS acts as co-receptors by facilitating the binding of FGF ligands to FGFRS (Figure 12 B) [47, 85]. Intracellularly, HSPG has been reported to co-localize with FGF2 in the nucleus, suggesting that HSPG may act as a shuttle for the nuclear transport of the growth factor [86, 87]. We hypothesized that *EXT1*$^{-/-}$ cells have attenuated FGF signaling compared to WT cells.

First, I examined the activation of FGF/ERK signaling by doing Western blot for ERK (extracellular signal regulated kinase) and P-ERK. WT and *EXT1*$^{-/-}$ cells were differentiated into mesendoderm using CHIR protocol (Figure 3E) [74], cells were collected 1 day following CHIR treatment and lysed for Western blot. As a result, FGF signaling was attenuated in *EXT1*$^{-/-}$ cells with/without FGF2 stimulation (Figure 11A, 11B). Then, I attempt to rescue FGF signaling by adding varying concentrations of heparin back into the culture medium on a daily basis. Western blot showed that addition of heparin to the differentiation media could partially rescue FGF signaling activation (Figure 12 A). It is worth noting that Brachyury and P-ERK levels both peaked at 1 μg/mL, which is consistent with previous results and implicates a positive correlation between FGF/ERK signaling and Brachyury expression (Figure 12 A). Since the binding of FGF ligands to HS and FGFRs is critical for the initiation of downstream signal transduction, we hypothesized that cell-surface FGF binding is blocked in *EXT1*$^{-/-}$ cells. To test this hypothesis, undifferentiated cells were harvested and incubated with 1 μg/mL FGF2 prior to incubation with anti-FGF2 antibody. Cell-surface binding levels were detected by flow cytometry. While FGF binding was detected in WT cells, it was completely absent in the *EXT1*$^{-/-}$ cells (Figure 13 B). To corroborate this result, I also immunostained live WT and *EXT1*$^{-/-}$ cells for cell-surface FGF2. As

expected, there was no FGF2 signal observed in either undifferentiated or differentiated $EXT1^{-/-}$ cells (Figure 13 A). These results together indicated that lack of HS impedes the binding of FGF to cell surface and thereby attenuating FGF/ERK signaling pathway. In an attempt to rescue ME differentiation, I supplemented the differentiation medium with 10-25 ng/mL exogenous FGF2 (Figure 14 A). However, no significant recue effects were observed in the $EXT1^{-/-}$ cells for any FGF2 concentrations I tried (Figure 14 B).
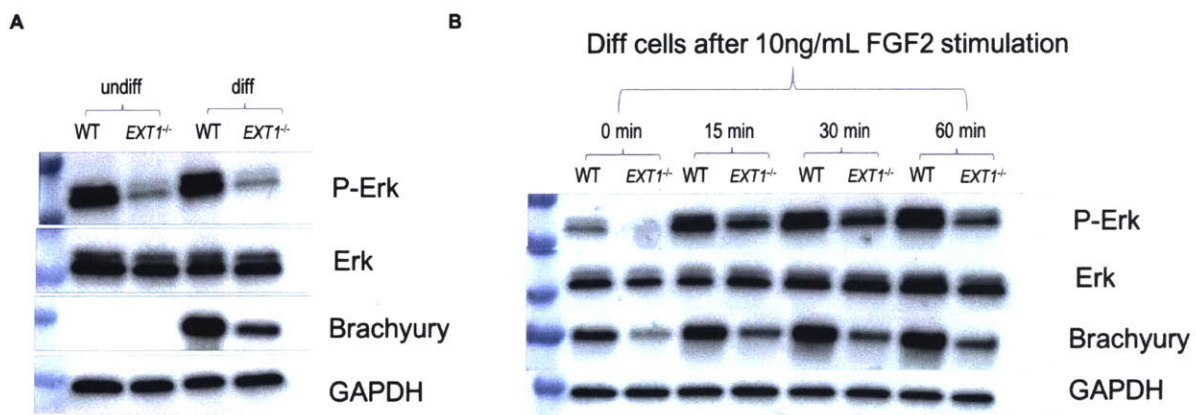
Figure 11. FGF/ERK signaling activation

(A) Protein level of Erk and P-Erk in WT and $EXT1^{-/-}$ cells at undifferentiated and differentiated states by Western blot (B) FGF/ERK signaling activation in differentiated WT and $EXT1^{-/-}$ cells following FGF2 stimulation. One day after ME differentiation, cells were stimulated with 10 ng/mL FGF2 and collected at 15, 30, 60 mins upon stimulation.
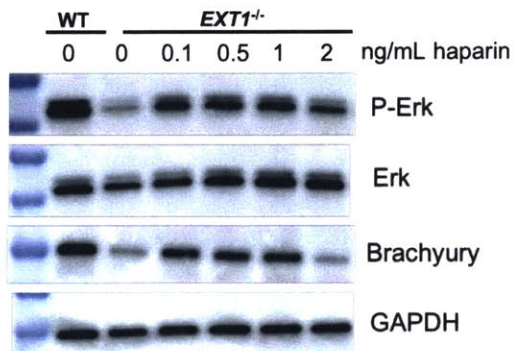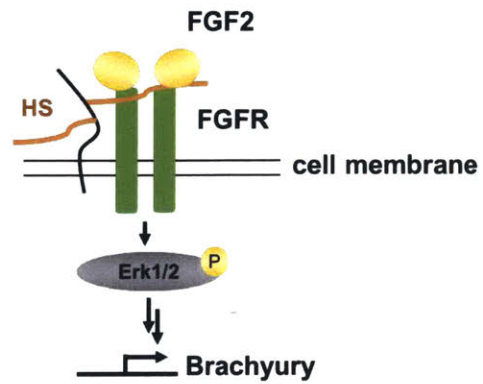
Figure 12. Heparin can partially restore the down regulation of FGF signaling in *EXT1⁻ᐟ⁻* cells (A) Detection protein level of Erk and P-Erk in differentiated WT and *EXT1⁻ᐟ⁻* cells by Western blot. *EXT1⁻ᐟ⁻* cells were supplemented with heparin ranging from 0.1 µg/mL-2 µg/mL during ME differentiation. (B) Proposed model of HS-FGFR2 interaction: HS increases the affinity of FGF2 to their receptors FGFR, stabilizing the ligand:receptor complexes thus facilitating downstream signaling activation.
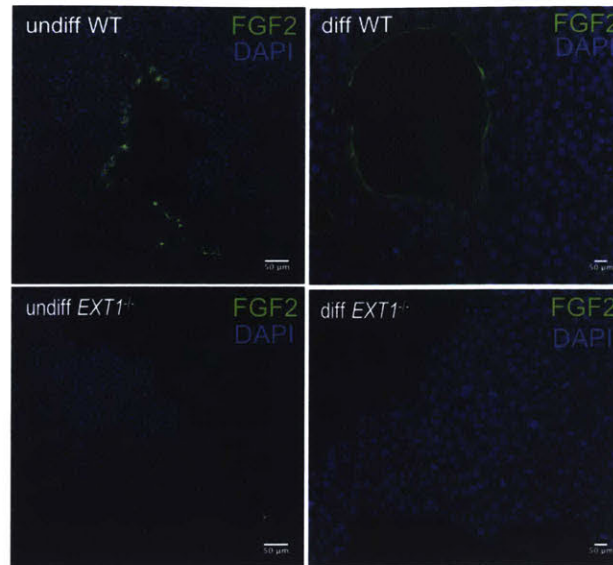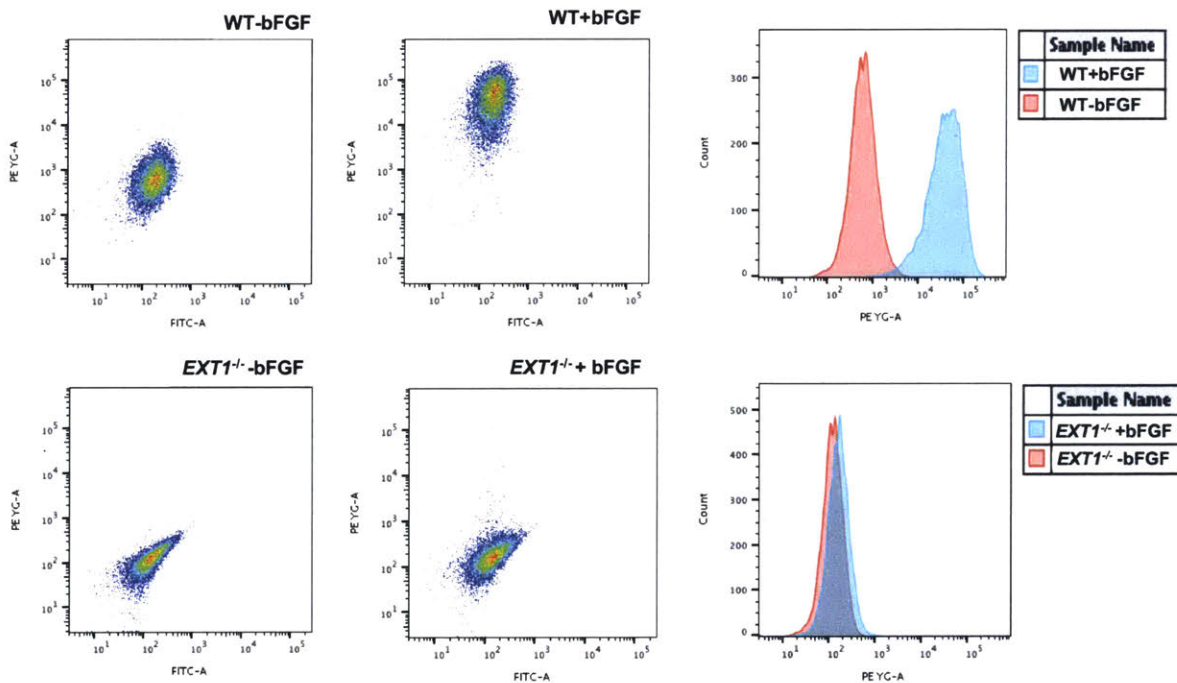
Figure 13. Comparing FGF binding affinity between WT and *EXT1⁻ᐟ⁻* cells
(A) Immunostainings of FGF2 (green) in WT and *EXT1⁻ᐟ⁻* cells at undifferentiated and differentiated states. Nuclei were counterstained with DAPI (blue). (B) Flow cytometry analysis of FGF2 binding in WT (WT) and *EXT1⁻ᐟ⁻* cells: WT and *EXT1⁻ᐟ⁻* cells were collected and incubated with or without 1µg/mL basic FGF (FGF2). PE-conjugated anti-bFGF antibody and live-dead stain were used for sample preparation. Individual dot plots are shown on the left.
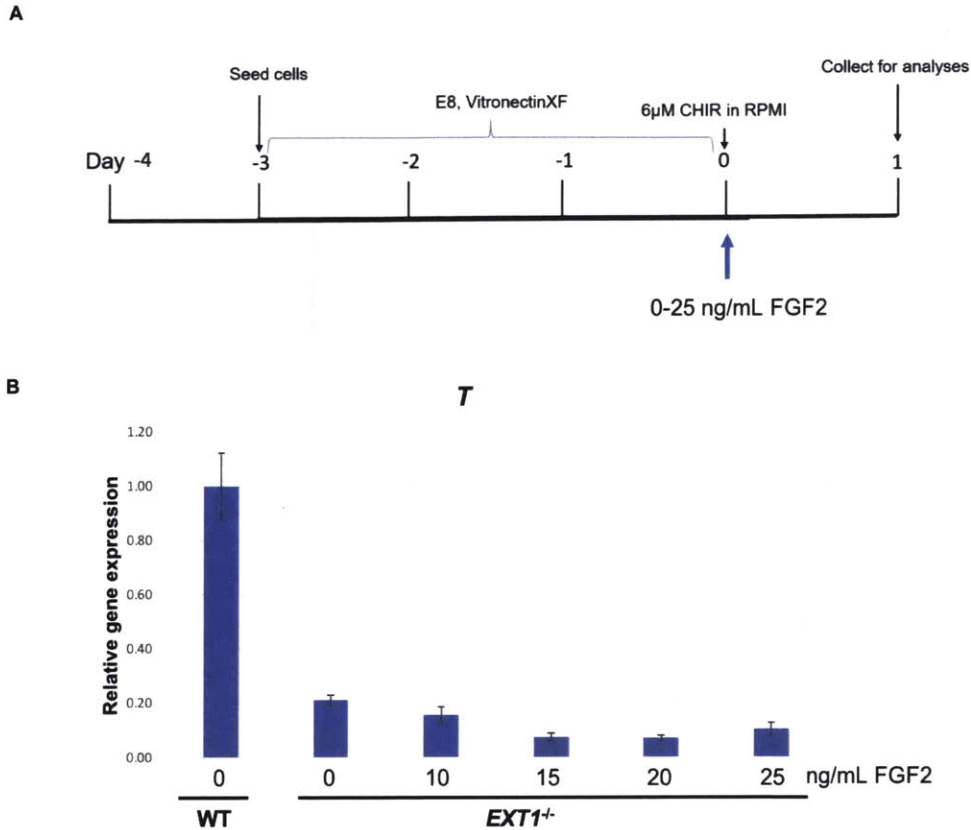
Figure 14. Determining if ME differentiation defect can be restored in $EXT1^{-/-}$ cells by exogenous FGF2

(A) Schematic diagram showing FGF2 complementation strategy. 0-25 ng/mL FGF2 was added in RPMI with 6uM CHIR to induce differentiation. (B) qPCR analysis of T expression in WT and $EXT1^{-/-}$ collected on day 1 of ME differentiation.

4.2. BMP signaling and Activin A signaling

BMP, and Activin A belong to the TGF-β (transforming growth factor-beta) superfamily [88-90]. Both signaling pathways are well-established important developmental signaling pathways for normal mesoderm and endoderm differentiation [88, 89]. TGF-β signaling pathways are initiated by ligand-receptor interactions followed up by the recruitment and phosphorylation of R-Smads (Smad1/5 for BMP, Smad2/3 for Activin A). These phosphorylated R-Smads then associate with the co-Smad, Smad4, to form an active complex, which in turn translocates into the nucleus to initiate transcription [90].

First, I sought to examine the degree of phosphorylation of Smad1/5 in WT and $EXT1^{-/-}$ cells by Western blot. Undifferentiated $EXT1^{-/-}$ cells showed more P-Smad1/5 than WT cells (Figure 15A), suggesting that BMP signaling is actually more activated in $EXT1^{-/-}$ cells at undifferentiated state. This is not very surprising given the role of BMP signaling in hPSCs is very context dependent. Although BMP sustains self-renewal of mouse embryonic stem cells, it induces differentiation in human embryonic stem cells [91]. Some previous work done in the lab revealed that $EXT1^{-/-}$ cells readily differentiated in bFGF-deficient conditions. It's possible that this high basal level of BMP signaling in $EXT1^{-/-}$ cell line contributes to its inability to effectively maintain in a pluripotent state. Moreover, differentiated $EXT1^{-/-}$ cells showed attenuated activation of BMP signaling with or without BMP4 stimulation (Figure 15A, 15B). Examination of Smad4 showed no significant change in Smad4 level between WT and $EXT1^{-/-}$ cells (Figure 15C). Addition of exogenous BMP4 (1-40 ng/mL) alone couldn't restore Brachyury expression in $EXT1^{-/-}$ cells (Figure 16A, 16B). These results suggested that HS-deficiency leads to an attenuated activation of BMP signaling in $EXT1^{-/-}$ cells exacerbating their differentiation defect.
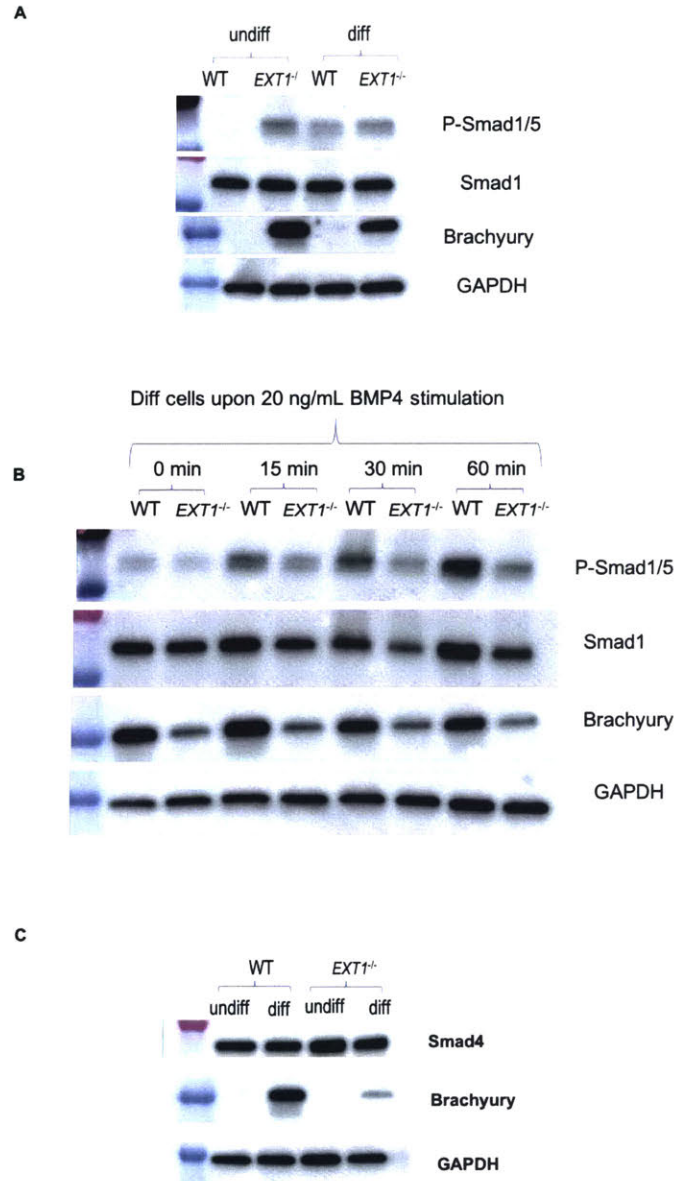
Figure 15. Comparing BMP signaling activation between WT and *EXT1*$^{-/-}$ cells

(A) Western blot of Smad1 and P-Smad1/5 in WT and *EXT1*$^{-/-}$ cells at undifferentiated and differentiated states. (B) Protein level of Smad1 and P-Smad1/5 in differentiated WT and *EXT1*$^{-/-}$ cells following BMP4 stimulation. 1 day after ME differentiation, both WT and *EXT1*$^{-/-}$ cells were stimulated with 20 ng/mL BMP4 and were collected at 15, 30 and 60 mins upon stimulation. GAPDH was used as a loading control. Brachyury was detected indicating successful ME differentiation. (C) Protein level of Smad4
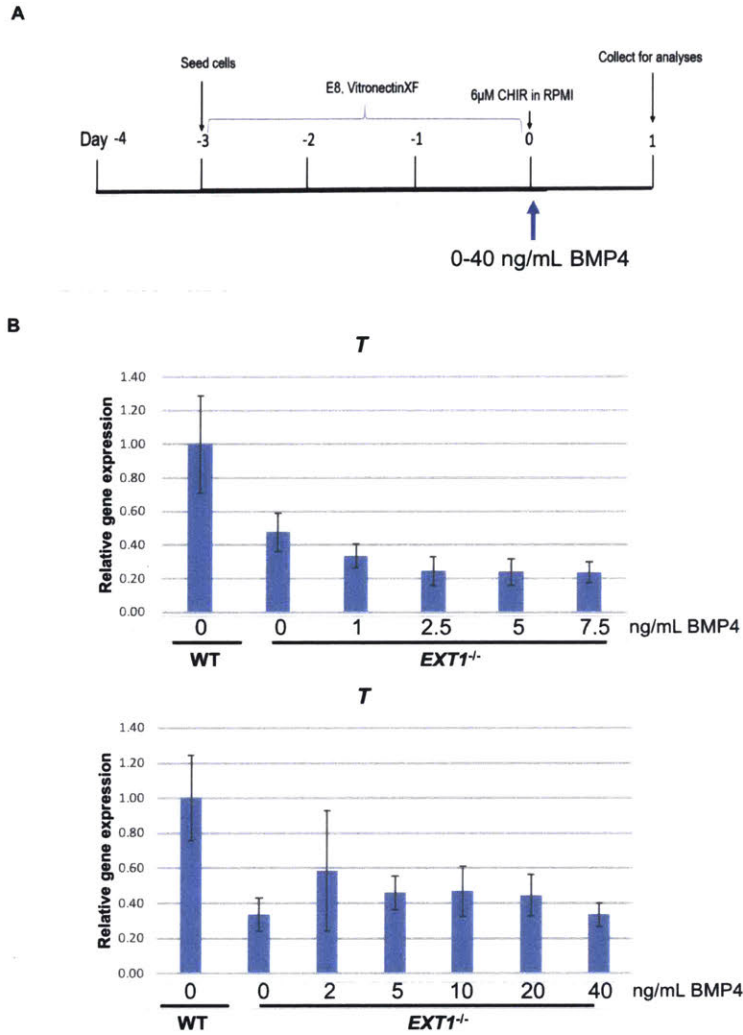
Figure 16. Testing if ME differentiation defect can be restored in *EXT1*⁻/⁻ cells by exogenous BMP4

(A) Schematic diagram showing BMP4 complementation strategy. 0-40 ng/mL BMP4 was added in RPMI with 6uM CHIR to induce differentiation. (B) qPCR analysis of *T* expression in WT and *EXT1*⁻/⁻ collected 1 day following ME differentiation.

Moving on to Activin A signaling, the protein levels of total Smad 2/3 and phosphorylated Smad 2/3 were examined by Western blot. Less phosphorylated Smad 2/3 was observed for *EXT1*⁻ᐟ⁻ cells in both undifferentiated and differentiated states with or without 10ng/mL Activin A stimulation (Figure 17A, 17B). Addition of exogenous Acitivin A (1-7.5 ng/mL) alone couldn't restore *T* or *MIXL1* expression in *EXT1*⁻ᐟ⁻cells upon ME differentiation (Figure 18A, 18B). These results suggested that HS-deficiency results in attenuated Activin A signaling in *EXT1*⁻ᐟ⁻cells similarly to its impacts on FGF and BMP signaling as mentioned above.
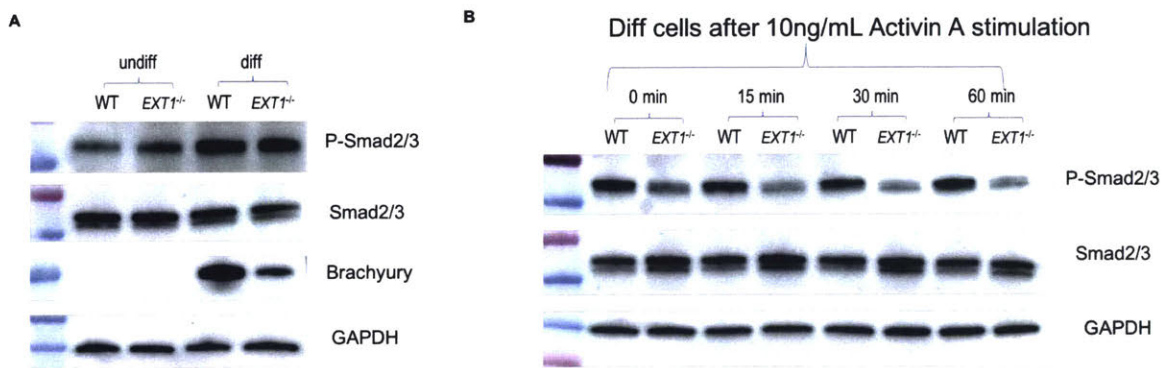
Figure 17. Comparing Activin A signaling activation between WT and $EXT1^{-/-}$ cells (A) Western blot of Smad2/3 and P-Smad2/3 in WT and $EXT1^{-/-}$ cells at undifferentiated and differentiated states. (B) Protein level of Smad2/3 and P-Smad2/3 in differentiated WT and $EXT1^{-/-}$ cells following Activin A stimulation. 1 day following ME differentiation, both WT and $EXT1^{-/-}$ cells were stimulated with 10ng/mL Activin A and were collected at 15, 30 and 60 mins upon. GAPDH was used as a loading control. Brachyury was detected indicating successful ME differentiation
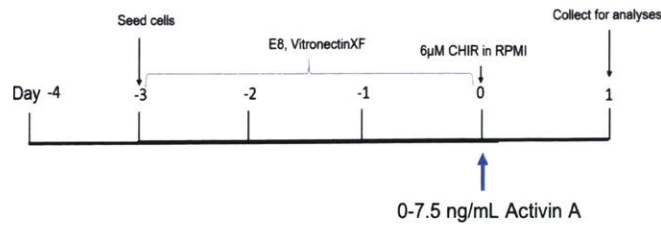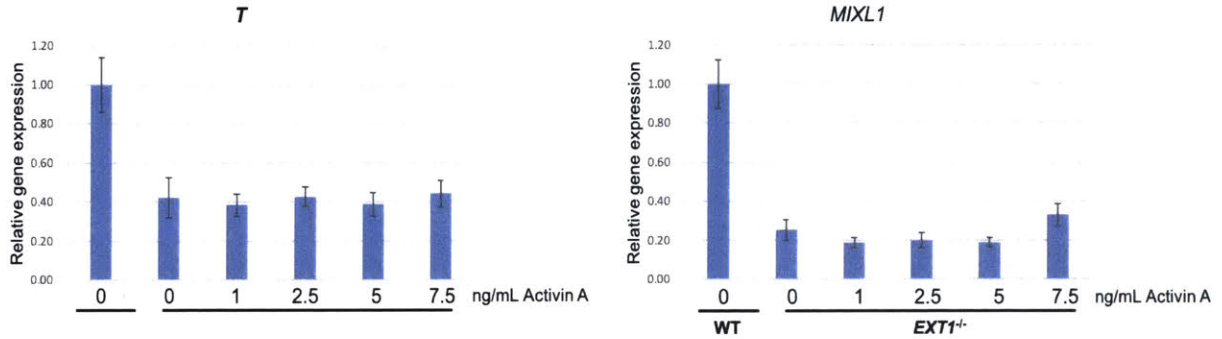
Figure 18. Testing if ME differentiation defect can be restored in *EXT1*^-/- cells by exogenous Activin A

(A) Schematic diagram showing Activin A complementation strategy. 0-7.5 ng/mL BMP4 was added in RPMI with 6uM CHIR to induce differentiation. (B) qPCR analysis of *T* and *MIXL1* expression in WT and *EXT1*^-/- collected 1 day following ME differentiation.

4.4. Wnt/β-catenin signaling

The Wnt/β-catenin pathways are initiated by the binding of Wnt ligands to Frizzled receptors on the cell membrane [92]. HSPGs function as cofactors for Wnt- Frizzled interactions [93-95]. In the absence of Wnt ligand, cytosolic β-catenin is phosphorylated and targeted for proteasomal degradation [92, 96]. When Wnt ligand binds to Frizzled receptors, a protein Dishevelled (DVL) is recruited to the membrane, preventing the phosphorylation of β-catenin, which can translocate into the nucleus to initiate transcription [92, 95, 96]. I examined canonical Wnt signaling activation by doing a Western blot for the unphosphorylated β-catenin (active β-catenin). No remarkable difference was observed in active β-catenin at protein level between WT and *EXT1*^-/- cells (Figure 19). However, not only is β-catenin involved in canonical Wnt signaling, it also functions in cadherin-associated cell adhesion (adherens junction) [97]. So, the total active β-catenin tested here comprised β-catenins involved in both cell adhesion and canonical Wnt

signaling. Because of this, I was unable to draw any conclusion from this Western blot regarding the activation level of canonical Wnt signaling. A preious mouse study showed that the activation of canonical Wnt signaling seems to remain unchanged in $EXT1^{-/-}$ mouse embryonic stem cells following ME differentiation [78]. Despite this, we still cannot predict that its activation level remains unchanged in our $EXT1^{-/-}$cells knowing that significant differences exist in the transcriptional networks and signaling pathways that control mouse and human ESC self-renewal and lineage development [21]. There are some other experiments that can be done to monitor Wnt/β-catenin signaling pathway. One is to test the protein levels of other critical constituents of the Wnt/β-catenin pathway such as DVL, which is an activator of Wnt/β-catenin signaling conveying signals from Frizzled receptors to the downstream effectors; and APC (adenomatous polyposis coli), which is a suppressor of the pathway by targeting β-catenin for degradation [96]. The second experiment is to carry out luciferase reporter assay to measure Wnt-reporter activity [98].
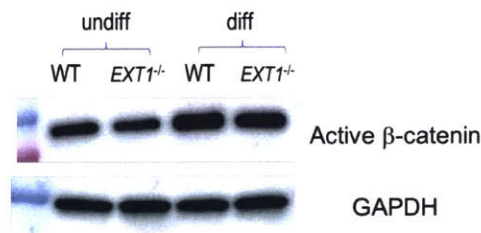
Figure 19. Protein level of Active β-catenin in WT and $EXT1^{-/-}$ cells at undifferentiated and differentiated states

4.5. Summary and perspective

In summary, $EXT1^{-/-}$ cells showed attenuated FGF, BMP and Activin A signaling upon ME differentiation. Addition of heparin could partially restore FGF/ERK signaling and Brachyury expression. However, Brachyury expression couldn't be rescued with FGF, BMP or Activin A alone. A well-formulated cocktail of all three growth factors is probably required to restore any ME differentiation in the $EXT1^{-/-}$ cells. Additional work should be done to test the cell-surface binding of BMP4 and Activin A. It would be also worth checking whether HS deficiency could affect the other three distinct pathways initiated by FGF-FGFR interaction: the Janus

kinase/signal transducer and activator of transcription (Jak/Stat), phosphoinositide phospholipase C (PLC) and phosphatidylinositol 3- kinase (PI3K) pathways [99].

Chapter 5:
Transcriptome profiling of *EXT1*^{-/-} cells

5.1. Overview of RNA-Seq

Although HS is well known for its roles in signaling pathways such as FGF and BMP [38], it has remained nascent what other kinds of signaling pathways are governed by HS. RNA-seq is a powerful tool because it can provide a comprehensive, unbiased view of cellular response to HS deficiency [100]. It is a great way to reinforce pre-existing knowledge while at the same time illuminating previously unknown HS-related transcripts or signaling pathways, based on which additional work can be carried out.

5.2. Sample preparation

Preparing samples for RNA-seq is almost the same as preparing samples for regular qPCR analysis, except I did some tests to pinpoint the time point at which the largest difference in Brachyury expression between WT and *EXT1*^{-/-} cells emerges. 500,000 cells were seeded and cultured for three days in E8 on vitronectin prior to treatment with 6 μM CHIR in RPMI (Figure 3E). Cells were then collected at 20 hr, 24 hr, 28 hr, 32 hr, or 36 hr following treatment and Brachyury expression was evaluated for each time point. As is shown in the qPCR and flow cytometry analysis, Brachyury expression increased from 20 hr, 28 hr to 32 hr and went down afterwards (Figure 20A, 20B). The largest difference in Brachyury expression seemed to happen between 24 and 28 hours. At 24 hr, 70.1% of the WT cells were Brachyury-positive and 1.2% of the *EXT1*^{-/-} cells were Brachyury-positive. At 28 hr, 75.9% of the WT cells were Brachyury-positive and 1.5% of the *EXT1*^{-/-} cells were Brachyury-positive (Figure 20A). Given that the difference in Brachyury transcript level seemed to be greater at 28 h (Figure 20B)., I chose to submit samples of WT and *EXT1*^{-/-} cells harvested after 28 hours of differentiation with 4 replicates of each. Samples of undifferentiated cells were prepared and submitted by Dr. Masuko in the lab.

In brief, 16 samples were submitted with 4 replicates of each condition:
H9_UNDIFF1a, H9_UNDIFF2a, H9_UNDIFF3a, H9_UNDIFF4a;
EXT1_UNDIFF1a, EXT1_UNDIFF2a, EXT1_UNDIFF3a, EXT1_UNDIFF4a;

H9_DIFF1a, H9_DIFF2a, H9_DIFF3a, H9_DIFF4a;

EXT1_DIFF1a, EXT1_DIFF2a, EXT1_DIFF3a, EXT1_DIFF4a;
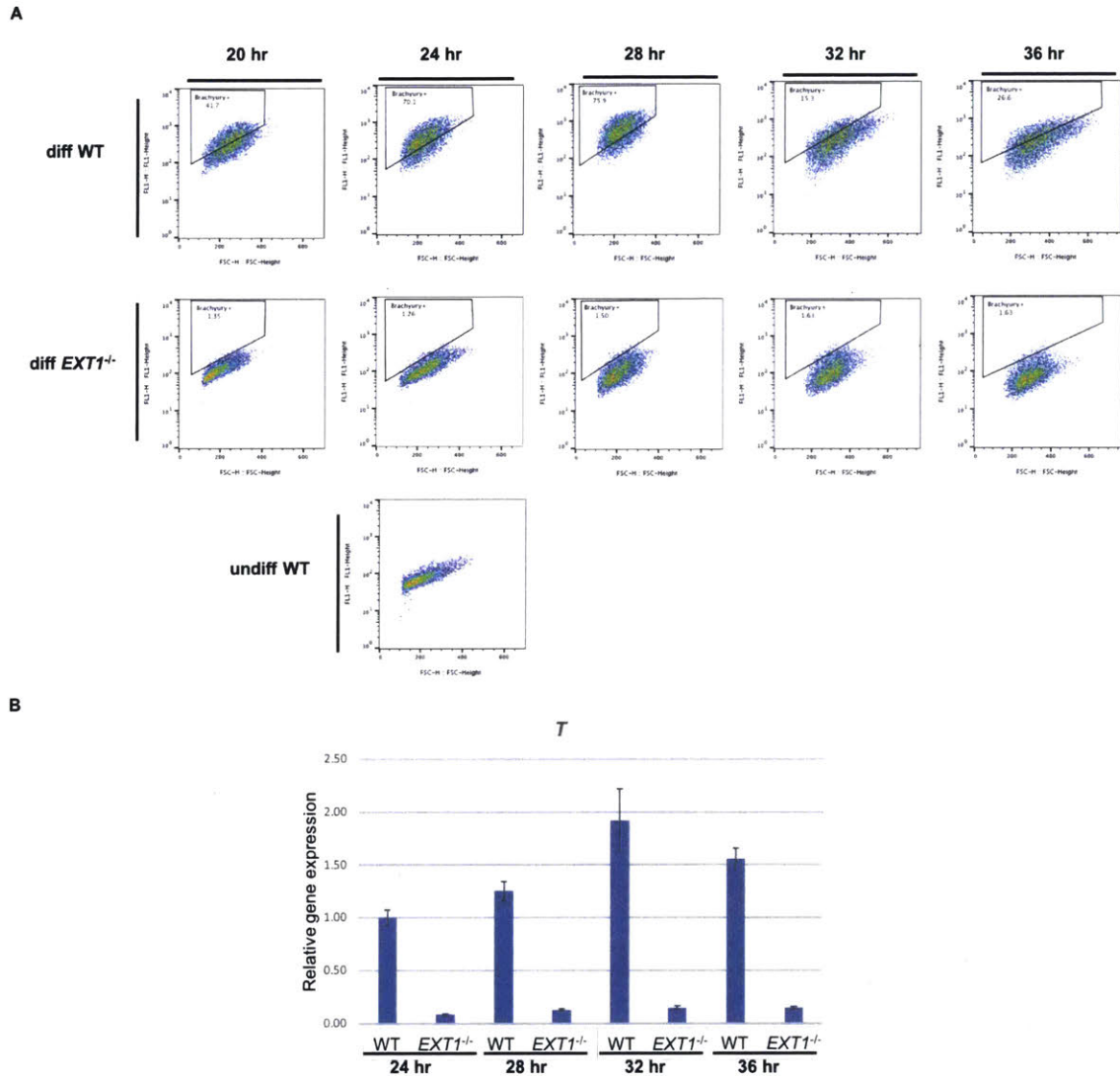
Figure 20. Selecting the time point for the largest difference in *T* expression between differentiated WT and *EXT1⁻ᐟ⁻* cells for RNA-Seq sample submission

(A) Flow cytometry analysis of Brachyury. Cells were collected at 20, 24, 28, 32 and 36 hours upon ME differentiation induction. Undifferentiated WT cells at 24hr is served as a negative control. (B) Gene expression level of *T* in differentiated WT and *EXT1⁻ᐟ⁻* cells collected at 24, 28, 32 and 36 hours.

5.3. Results

Four RNA-seq comparisons were created:

UNDIFF_EXT1$^{-/-}$_UNDIFF_H9 comparison, DIFF_EXT1$^{-/-}$_DIFF_H9 comparison,

DIFF_H9_UNDIFF_H9 comparison and DIFF_EXT1$^{-/-}$_UNDIFF_ EXT1$^{-/-}$ comparison

Since I'm more interested in the transcriptional changes between WT and EXT1$^{-/-}$ at differentiated state, I'll focus on the DIFF_EXT1$^{-/-}$_DIFF_H9 comparison in this thesis. From the Differential Gene Expression (DGE) report for the DIFF_EXT1$^{-/-}$_DIFF_H9 comparison, 15,811 genes were identified in total, among which 4144 genes showed differential expression (DGE) with statistical significance. Among the 4144 differentially expressed genes, 1996 genes were significantly upregulated and 2148 were significantly downregulated.

To evaluate how confident we can be in the results of the DGE analysis, I checked whether the 4 replicates of the same condition clustered together or whether they were separated from replicates of other conditions because large within-group variance is generally indicative of problems with upstream processing procedures (*e.g.* sample collection, batch effects, etc.). An unsupervised multidimensional scaling (MDS) plot is a visualization of a principle components analysis and shows similarities and dissimilarities between samples as sources of variation in the data [101]. Ideally, each factor in the MDS plot will cluster well within the primary condition of interest and be separated from other conditions. This indicates that differences between groups (effect size) are larger than differences within groups [101]. An inspection of the MDS plot generated for all 16 samples showed that samples of the same condition clustered well together (Figure 21), suggesting that the experiment was well controlled. It is worth noting that H9_DIFF1a is more distant from the other 3 H9_DIFF samples and EXT1_UNDIFF3a is a little far away from the other 3 members of the same group probably owing to batch effects or true biological variation. Moreover, I checked whether the RNA-seq results showed evident differential gene expression in a specific set of transcripts known previously to differ in abundance among treatment groups determined by qPCR. Multiple primitive streak/mesoderm markers: *T, MIXL1, NODAL, EOMES* were all significantly downregulated by about 2-fold in the RNA-seq data, which is coherent with previous qPCR results. Therefore, I concluded that the RNASeq experiment was a "success" and proceeded to analysis.

41
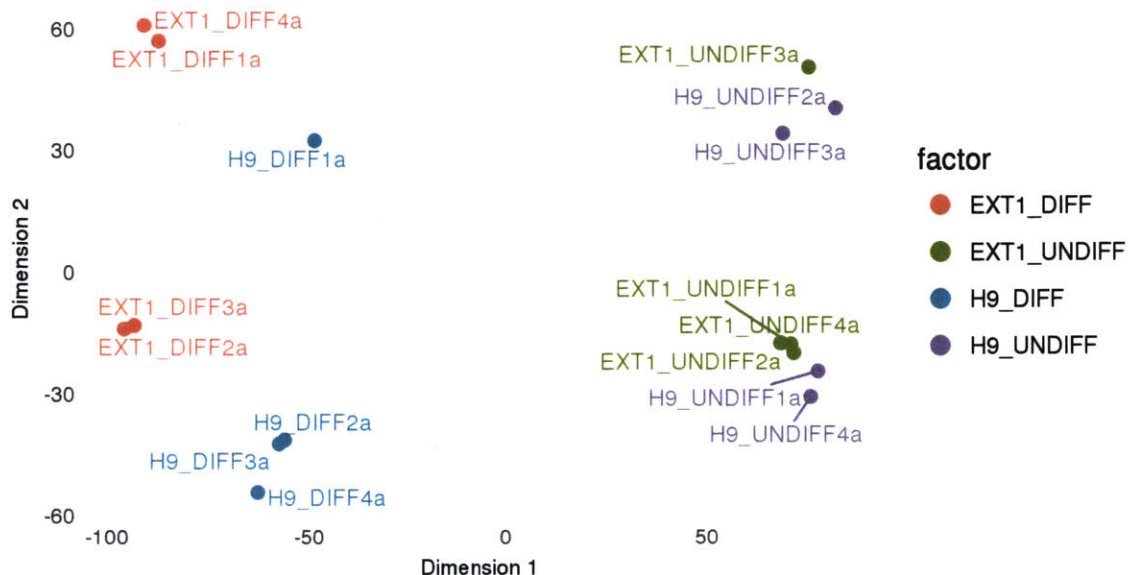
Figure 21. MDS plot for all samples together at the gene level

Distances on the plot may be interpreted as leading $\log_2$ fold-change, meaning the $\log_2$ fold-change between the samples for the genes that distinguish those samples. It is expected that the between-group variance of gene expression is greater than the within-group variance and therefore can be detected.

5.4. Canonical pathway analysis

With the recent and rapid advance of RNA-Seq analysis tools, the analysis and interpretation of RNA-seq data is far from routine. In this case, the goal of my RNA-seq analysis is to answer two major questions: (1) What is known about the differently expressed genes in our data and which pathways/biological themes are the most significant? (2) Can I associate our RNA-seq results to any human diseases and if HS defect can potentially contribute to that specific disease? To answer the first question, I took advantage of Ingenuity Pathway Analysis (IPA) Core Analysis to identify the most relevant signaling and metabolic pathways, molecular networks, and biological functions for all 4144 DGE genes. Some of the top hits were summarized in Figure 22. hESC pluripotency and transcriptional regulatory networks were significantly differentially regulated indicating that *EXT1*^{-/-} cells have self-renewal and differentiation defect. HSPGs are known to act as coreceptors for the Wnt, FGF, Hedgehog (Hh), and BMP pathways [20, 48, 102]. Among these

growth factor signaling pathways, FGF and TGF-β signaling were significantly downregulated, which is consistent with previous observations. PCP (Wnt/planar cell polarity), Wnt/β-catenin signaling and Sonic Hedgehog signaling were significantly upregulated in $EXT1^{-/-}$ cells. This is consistent with a previous study showing that Wnt signaling was elevated in $EXT1^{-/-}$ mouse cells that had ME differentiation defect [78]. The PCP signaling pathway is crucial for polarized cell migration during gastrulation and neural tube closure [103-105]. Previous studies showed that syndecan-4 regulates the Wnt/PCP pathway in Xenopus embryos [103, 106] and syndecan-4 knockout mice display a hallmark phenotype of disturbed non-canonical Wnt [105]. Hence, it would be interesting to compare the activation of PCP pathway between WT and $EXT1^{-/-}$ cells in more details. Hedgehog signaling is required for the differentiation of human embryonic stem cells into neurectoderm [107]. Specifically, removing Hedgehog signaling in neural crest cells results in abnormal patterning and growth of facial primordia [108]. Increased Sonic Hedgehog signaling is consistent with the observations we have from the RNA-seq data that $EXT1^{-/-}$ cells upregulated a lot of genes involved in neurogenesis and neural crest stem cell development. It is also known that Hedgehog signaling plays an important role in bone formation [109], so upregulated sonic Hedgehog signaling in $EXT1^{-/-}$ cells may be a cause for aberrant chondrocyte proliferation which results in formation of exostoses in patients with HME. Notably, a couple of the signaling pathways involved in inflammatory responses were among the top hits of canonical pathways identified by IPA Core Analysis: osteoarthritis pathway, C-X-C chemokine receptor type 4 (CXCR4) signaling, JAK family kinases in IL-6-type cytokine signaling, IL-1 signaling and NF-κB signaling, highlighting the roles of HS in regulating the inflammatory and immune responses. It is known that HS interacts with many components of the immune system [110, 111]. In fact, a bioinformatics analysis identified 235 candidate proteins in the mouse immune system that can potentially interact with mammalian proteins that express a heparin/HS-binding motif [110]. HS is known to interact with Toll-like receptor 4 in the innate immune system, the activation of which leads to the activation of NF-κB signaling pathway and inflammatory cytokine production [112, 113]. It is proposed that HS may recognize the cytoplasmic domains of Toll-like receptors and modulate the binding between Toll-like receptors and their cognate ligands [112]. Furthermore, HS can associate with the basement membrane to form a physical barrier, and during inflammation, infiltrating monocytes and neutrophils degrade this HS barrier to help their extravasation [113, 114]. Gonadotropin-releasing hormone (GRH) signaling was
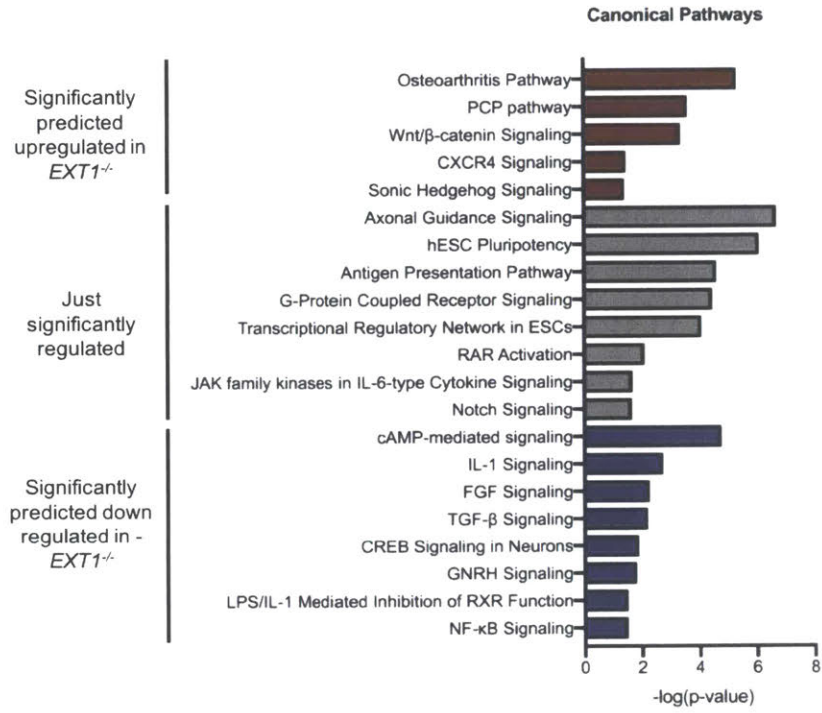
43

significantly downregulated in $EXT1^{-/-}$ cells. It is known that anosmin-1 (encoded by gene ANOS1) can interact with cell-membrane bound HSPGs to modulate FGFR 1 signaling in GnRH neurons [115]. And the gene expression of ANOS1 was significantly downregulated by about 4-fold in the differentiated $EXT1^{-/-}$ cells in our RNA-seq data. But how exactly HS is involved in GRH signaling awaits more investigation. It is worth noting that cAMP-mediated signaling was significantly down regulated in $EXT1^{-/-}$ cells with the smallest Z-score among all the 21 canonical pathways summarized here. In the same line with cAMP-mediated signaling, G-protein coupled receptor signaling was also differentially regulated with statistical significance, indicating $EXT1^{-/-}$ cells probably had impaired G-protein-coupled receptor-mediated cAMP signaling. HS has been shown to interact with 7-helix transmembrane receptors coupled to G-protein [116, 117]. For example, HS can bind chemokines and facilitate their interactions with chemokine receptors, 18 of which belong to the G protein-coupled receptor (GPCR) family [116-119]. But it would be worth investigating what other GPCRs HS interacts with in processes outside the immune system and how that could relate to defect associated with HS deficiency. Importantly, retinoic acid receptor (RAR) activation was differentially regulated with statistical significance in $EXT1^{-/-}$ cells. While RAR signaling is indispensable for motor neuron differentiation, it is not required for neural crest stem cell differentiation from neural progenitor cells [120-123]. It has been discovered previously in the lab that $EXT1^{-/-}$ cells were able to differentiate into neural crest stem cells but not into motor neurons. Therefore, this piece of RNA-seq data supports our experiment results and provides possible explanations that $EXT1^{-/-}$ cells were unable to differentiate into motor neurons because of impaired RAR activation.

5.5. Match analysis

To answer the second question as to whether a human disease can be linked to our RNA-seq result, I took advantage of IPA Match Analysis function to match existing IPA analysis data of human diseases to the core analysis results of the DIFF_EXT1$^{-/-}$_DIFF_H9 comparison. IPA analyses were identified and scored based on shared patterns of signatures. Unfortunately, there is no RNA-seq data related to HME available at the moment for us to compare our data with. Among the top hits, I selected two IPA analyses that seemed intriguing to me for discussion here. It appeared that the transcriptional changes in differentiated $EXT1^{-/-}$ compared to differentiated WT are comparable to gene expression changes in colonic mucosal samples collected from

patients with Crohn's disease (NCBI GEO: GSE52746) [124] and Ulcerative colitis (NCBI
GEO: GSE50594) (unpublished study) (Figure 23). As is shown in the heatmaps, a lot of
upstream regulators (indicated by UR) are shared between DIFF_EXT1$^{-/-}$_DIFF_H9 and Crohn's
disease/Ulcerative colitis, many of which are components of the inflammatory and immune
reactions such as IFNG (interferon gamma), IFNA1/IFNA13 (interferon alpha 1), IFNL1
(interferon lambda 1), TLR3 (toll like receptor 3), etc. (Figure 23) Crohn's disease and ulcerative
colitis are chronic forms of inflammatory bowel disease (IBD) [125]. Interestingly,
immunohistochemical analysis showed that no heparanase expression was detected in in
specimens derived from normal colon tissue, whereas strong heparanase staining was detected in
Crohn's disease and ulcerative colitis [126]. Studies on mice with an IBD-like gut disorder
revealed altered expression of syndecan-1 and -4 and HS in the gut [127]. A marked reduction of
syndecan-1 was also observed in reparative epithelium from patients with IBD [128]. Moreover,
heparin has been shown to aid healing in ulcerative colitis [128, 129]. All these findings imply
that HS might have a protective effect against inflammatory bowel disease (IBD). Despite the
differences in treatment, cell type and even control group, our RNA-seq data for the
DIFF_EXT1$^{-/-}$_DIFF_H9 comparison shared a lot of similar features with data obtained from
Crohn's disease and ulcerative colitis.

A

Canonical Pathways

Significantly predicted upregulated in *EXT1⁻ᐟ⁻*

- Osteoarthritis Pathway
- PCP pathway
- Wnt/β-catenin Signaling
- CXCR4 Signaling
- Sonic Hedgehog Signaling

Just significantly regulated

- Axonal Guidance Signaling
- hESC Pluripotency
- Antigen Presentation Pathway
- G-Protein Coupled Receptor Signaling
- Transcriptional Regulatory Network in ESCs
- RAR Activation
- JAK family kinases in IL-6-type Cytokine Signaling
- Notch Signaling

Significantly predicted down regulated in - *EXT1⁻ᐟ⁻*

- cAMP-mediated signaling
- IL-1 Signaling
- FGF Signaling
- TGF-β Signaling
- CREB Signaling in Neurons
- GNRH Signaling
- LPS/IL-1 Mediated Inhibition of RXR Function
- NF-κB Signaling

-log(p-value)

B

Canonical Pathways

- Sonic Hedgehog Signaling
- PCP pathway
- CXCR4 Signaling
- Osteoarthritis Pathway
- Wnt/β-catenin Signaling
- LPS/IL-1 Mediated Inhibition of RXR Function
- FGF Signaling
- cAMP-mediated signaling
- NF-κB Signaling
- CREB Signaling in Neurons
- IL-1 Signaling
- TGF-β Signaling
- GNRH Signaling

z-score

46

Figure 22. Pathway Analysis of diff *EXT1⁻ᐟ⁻*_diff WT comparison

(A) Canonical pathways significantly upregulated (red) and downregulated (blue) (Fisher's exact test, $-\log10$ P values for each represented pathway are shown) in transcripts in *EXT1⁻ᐟ⁻* from diff*EXT1⁻ᐟ⁻*_diff WT comparison, as predicted by Ingenuity Pathway Analysis (IPA). Pathways in gray are significant but without known degree of functional activation/inhibition. (B) Z-scores for canonical pathways significantly upregulated (red) and downregulated (blue) as predicted by IPA. Z-score reflects the overall predicted activation state of the biological function (<0: decreased, >0: increased). Positive Z-score indicates upregulated pathways and negative z-score represents downregulated pathways. A higher absolute value of z-score indicates a pathway has more "activated" predictions (z>0) or more "inhibited" predictions (z<0).
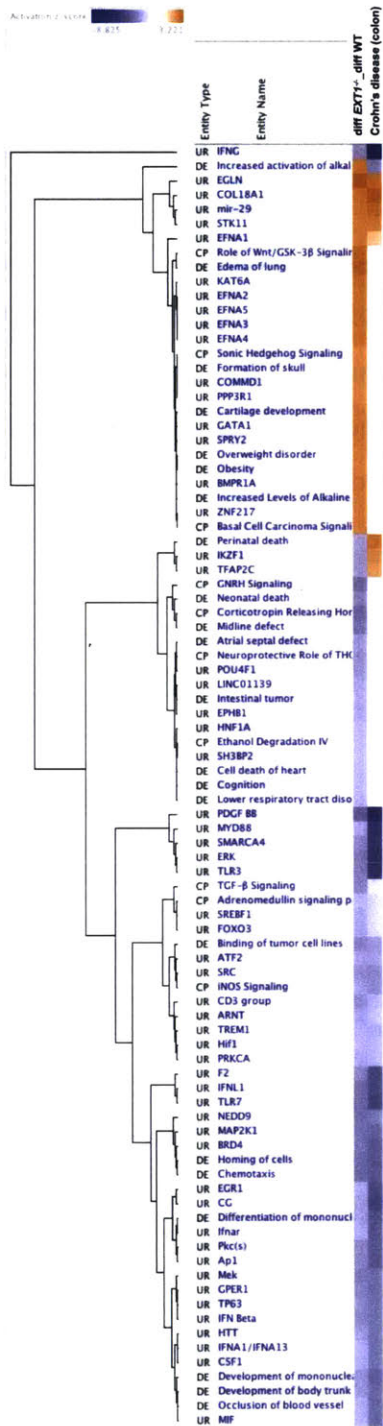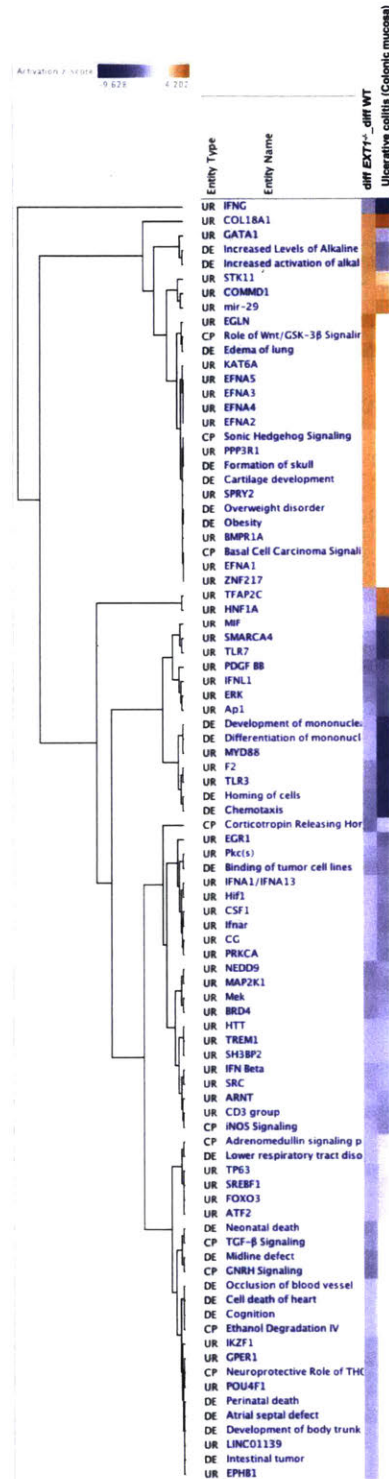
Figure 23. Analysis match of diff EXT1-/-_diff WT comparison with comparative datasets of human diseases

IPA Core Analyses of diff EXT1-/-_diff WT comparison are matched to over 47,000 IPA analyses that have been processed from public sources using Array Suite. Two IPA analyses are identified based on shared patterns of signatures. The entities of the signatures (the rows) versus the analyses (the columns) selected are shown in heatmaps. A is diff $EXT1^{-/-}$_diff WT comparison versus Crohn's disease (colon). B is diff $EXT1^{-/-}$_diff WT comparison versus ulcerative colitis (colonic mucosa). The entities that make up each signature for the analysis (the rows) are clustered by default. The cells in the heatmap are colored to indicate the z-score for the entity in the analysis. Orange squares have a positive z-score and blue squares have a negative z-score. Entity Types are classified into: UR (upstream regulators), CN (causal network master regulators), DE (diseases and functions) and CP (Canonical Pathways).

5.6. Neural crest stem cell differentiation potential of $EXT1^{-/-}$ cells

Neural crest stem cells (NCSCs) are multipotent embryonic cells that can give rise to cells of the ectodermal lineage, such as neurons, and even cells of the mesodermal lineage such as cardiac cells, chondrocytes and melanocytes [130, 131]. NCSCs originate at the border of the neural plate and at the dorsal aspect of the neural tube. Early neural crest development occurs in two phases from gastrulation to neurulation [132]. During gastrulation, early neural crest stem cell development goes through two stages: neural plate border (NPB) specification and neural crest (NC) induction. NPB specification is induced by most notably BMP, FGF, and Wnt signaling pathways [132-135]. A set of regulatory transcription factors has been well-established as NPB specifiers (Msx1/2, Pax3/7, Zic1, Dlx3/5, Hairy2, Id3, Ap2) [132, 133, 136, 137]. As development progresses, neural plate border (NPB) goes through neural crest (NC) induction, where the expression of a second set of transcription factors named definitive neural crest (NC) markers (Snail2, FoxD3, Sox9/10, Twist, cMyc, and Ap2) are triggered [132, 133, 138, 139]. It is believed that these definitive NC markers would ultimately guide and control the completion of neural crest differentiation.

Although the ME development was suppressed, plenty of genes involved in neural crest development were significantly upregulated in the $EXT1^{-/-}$ cells from the RNA-seq data upon ME differentiation induction. This interesting observation made us wonder whether $EXT1^{-/-}$ cells are more prone in differentiate into neural crest cells (NSC) using NSC differentiation as a "coping

mechanism" when they are unable to undergo efficient ME differentiation. 6 genes (*HAND1*, *MSX1*, *PRDM1*, *PAX3*, *ZIC1*, *DLX5*) relevant to NSC development were among the top hits identified for the DIFF_EXT1$^{-/-}$_DIFF_H9 comparison and were selected for testing by qPCR (Figure 24A). As expected, qPCR analysis showed that most of the genes were upregulated with statistical significance, among which *ZIC1* and *PAX3* were increased by about 20-fold in *EXT1*$^{-/-}$ cells upon ME differentiation (Figure 24A). As mentioned above, *ZIC1*, *PAX3*, *DLX5, MSX1* are all critical neural plate border (NPB) specifiers [132, 133, 136, 137], it is safe to conclude that *EXT1*$^{-/-}$ cells upregulate markers of neural plate border (NPB) specification upon ME differentiation.

Next, I determined whether cell surface markers of neural crest stem cells (e.g., P75, HNK1) were expressed in WT or *EXT1*$^{-/-}$ cells following ME differentiation treatment. Little or no expression of P75 or HNK1 was observed in either WT or *EXT1*$^{-/-}$ cells by immunostaining or flow cytometry (Figure 24B, 24C). These results indicated that although *EXT1*$^{-/-}$ cells have the propensity to differentiate into neural crest stem cells, ME differentiation is not sufficient for them to become P75 and HNK1 double positive neural crest stem cells.
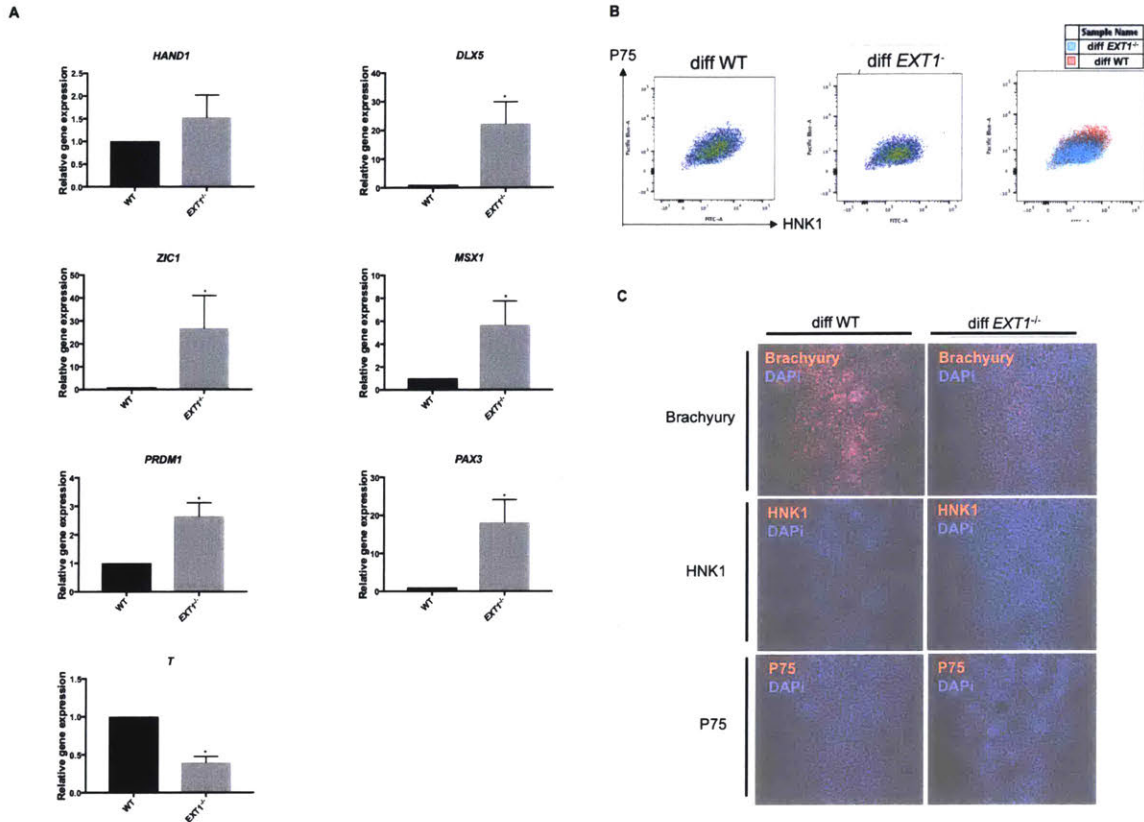
Figure 24. Comparing the expression of neural crest stem cell markers between differentiated WT and *EXT1*⁻/⁻ cells (A) Gene expression level of genes relevant to neural crest development in differentiated WT and *EXT1*⁻/⁻, * means p<0.05. (B) Flow cytometry analysis of cell surface marker P75 and HNK1 on differentiated WT and *EXT1*⁻/⁻ cells following one day of ME differentiation. (B) Immunostainings of differentiated WT and *EXT1*⁻/⁻ cells stained for Brachyury, HNK1 and P75 and counterstained for DAPI

5.7. Summary and perspective

Our RNA-seq result conforms that not only is ME differentiation hampered in *EXT1*⁻/⁻ cells, a lot of other biological processes are also affected significantly. Given that HS is found on cell surface, ECM, and intracellular compartments [19], it is not surprising to find that HS defect influences cellular activities at multifaceted levels. For instance, G-protein-coupled receptor-mediated cAMP signaling was significantly differentially regulated in *EXT1*⁻/⁻ cells indicating that HS may act as interacting partners of some cell-surface G-protein-coupled receptors. Upregulated inflammation process hints on the interactions between HS and various pro-inflammatory cytokines, suggesting that HS may be an important regulator in cellular infection

and immune response. These findings are of great significance in understanding the functions of HS at a more comprehensive level and open up new research avenues.

Analysis and interpretation of genomic data generated by sequencing technologies, such as RNA-seq, are among the most complex problems in genomic sciences. Different versions of software tools and specific commands can often produce very different results. Hence, we should always keep an open mind and revisit our RNA-seq data frequently whenever new questions or ideas arise.

Chapter 6:
Heparan sulfate deficiency and endoderm differentiation

6.1. Endoderm differentiation of WT and *EXT1*$^{-/-}$ cells

Previous findings in the lab highlighted the indispensable roles of HS in the ME and mesoderm differentiation of hESCs. To gain more insights into the functional roles of HS in definitive endoderm, I performed direct definitive endoderm (DE) differentiation using two strategies. In chemically-defined strategy, cells were treated with a cocktail of BMP4, Activin A and CHIR in E8 for 4 days prior to being collected for analysis of DE markers (Figure 25A). SOX17 (SRY-related HMG-box 17) and FOXA2 (forkhead box protein A2) are transcription factors and are required for normal development of the definitive gut endoderm [140, 141]. WT cells significantly upregulated SOX17 and FOXA2 upon differentiation into DE, whereas *EXT1*$^{-/-}$ cells didn't show any significant upregulation of SOX17 or FOXA2 (Figure 25B, 25C, 25E). Flow analysis of *SOX17* showed that around 33% of WT cells were *SOX17*-positive, which is around the average DE differentiation efficiency reported in other studies, however, only around 5% of *EXT1*$^{-/-}$ cells were *SOX17*-positive (Figure 25D). To further characterize these cells, I used hPSCs-derived endoderm qPCR arrays (Catalog # 07531, STEMCELL Technologies) to test the expression of 96 genes in *EXT1*$^{-/-}$ and WT cells upon DE differentiation (Figure 26). This qPCR array contained validated primers for detection of 90 genes whose expression is correlated with pluripotency or hPSC-derived ectodermal, mesodermal, and endodermal and endodermal lineage cells, including pancreatic, hepatic, and intestinal progenitor cells, as well as six endogenous (housekeeping) control genes. For sample preparation, *EXT1*$^{-/-}$ and WT cells were differentiated into DE using chemically-defined strategy and RNA were isolated and converted to cDNA via reverse transcription. For analysis, the expression profile of differentiated *EXT1*$^{-/-}$ cells was normalized to the expression profile of differentiated WT cells. And data was analyzed with an online qPCR analysis tool. In conclusion, median fold changes for the genes in the pluripotent, mesoderm, anterior endoderm, early endoderm, pancreas, live and intestine groups were below 1, indicating these biological processes were downregulated in *EXT1*$^{-/-}$ cells (Table 2). Among all the functional groups, early endoderm had the lowest median fold change (~0.35), suggesting it was greatly inhibited in *EXT1*$^{-/-}$ cells. Mesendoderm process was not obviously differentially

regulated with a median fold change being around 1. The median fold change for ectoderm process was slightly above 1 (~1.2) indicating it was enhanced in *EXT1⁻/⁻* cells (Table 2). A barplot was also generated to investigate individual gene expression levels of genes present in each group (Figure 27). Some of the greatly upregulated genes in *EXT1⁻/⁻* cells are *LCK, HEY1, LEFTY1, CDH1, T, ARX, SST, PAX6*, etc. (Figure 27) These results demonstrated that *EXT1⁻/⁻* cells have DE differentiation defect and they upregulate ectoderm markers in response to direct DE differentiation.
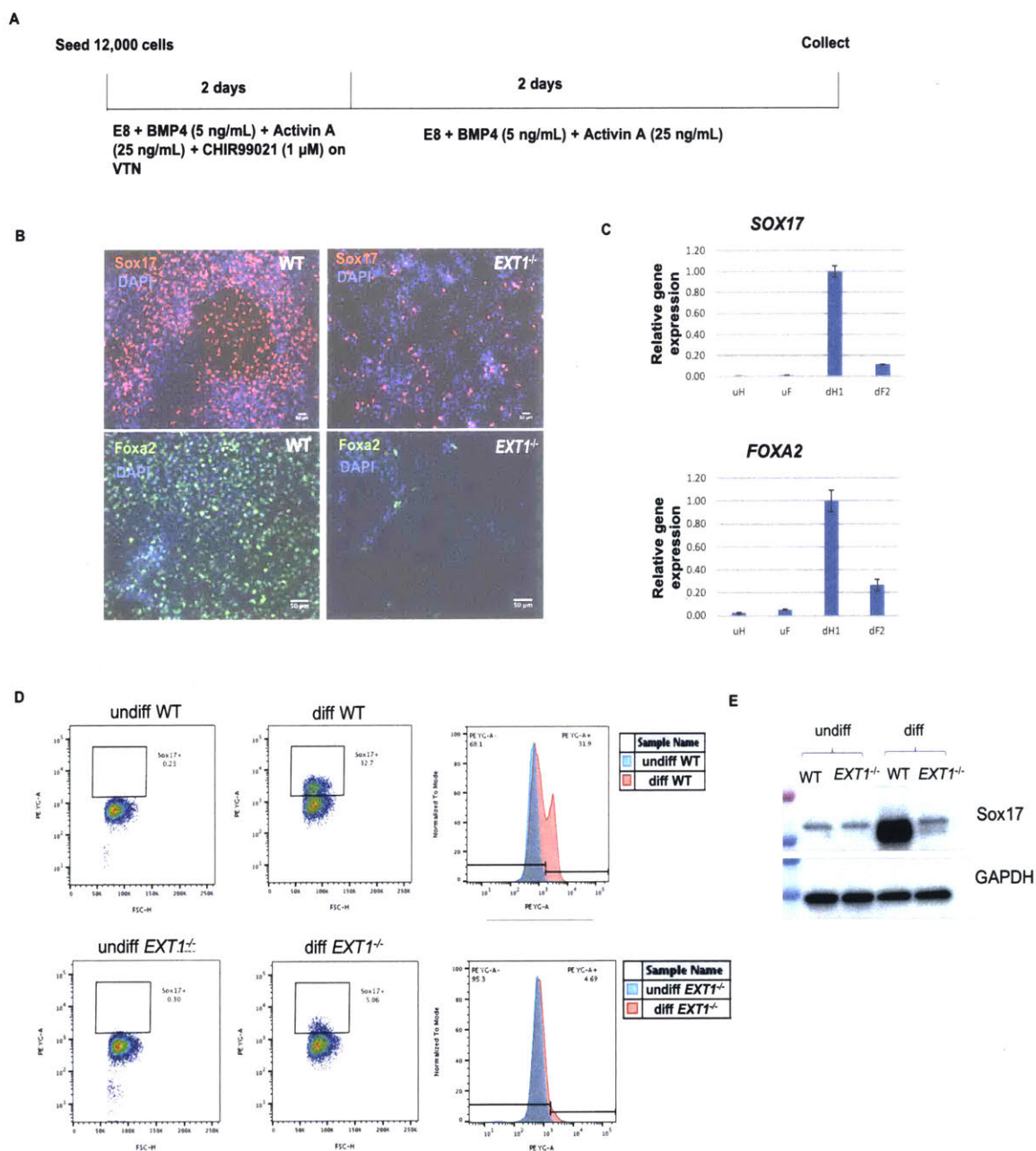
Figure 25. Chemically-defined endoderm differentiation of WT and *EXT1*⁻ᐟ⁻ cells

(A) Chemically-defined endoderm differentiation protocol. A cocktail of CHIR, BMP4 and Activin A in E8 was used to induce differentiation. (B) Immunostainings of differentiated WT and *EXT1*⁻ᐟ⁻ cells stained for Foxa2 (green) and Sox17 (red) and counterstained for DAPI (blue). (C) qPCR analysis of ED markers *SOX17* and *FOXA2* in WT and *EXT1*⁻ᐟ⁻ cells. (D) Flow cytometry analysis of Sox17 in WT and *EXT1*⁻ᐟ⁻ cells. The black box represents a gate for Sox17-positive events. A total of 100,000 individual events were collected. (E) Protein level of Sox17 using a polyclonal anti-Sox17 antibody and GAPDH as a loading control.
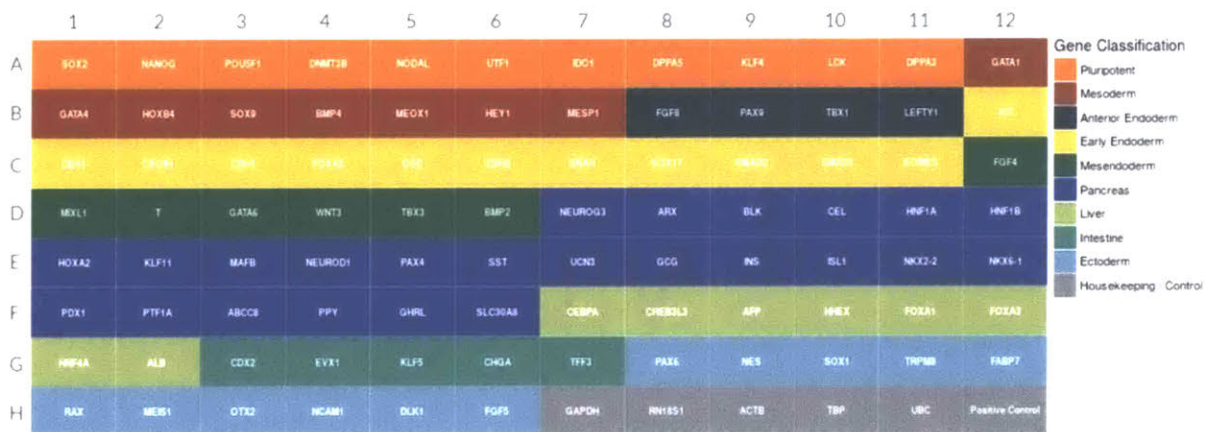
Figure 26. hPSCs-derived endoderm qPCR array plate configuration. A total of 96 genes are colored by the functional class of the genes. Housekeeping genes (gray) serve as controls.

Table 2. Global data table showing the results from endoderm qPCR array. Values are calculated based on gene expression changes between differentiated *EXT1*⁻ᐟ⁻ and WT cells. Downregulated gene categories have median fold change <1. A T-test (unpaired, two-tailed test with equal variance) is used for all statistical analysis. None of the fold changes are statistically significant because only one replicate has been tested.

Classification	Median Fold Change	pValue
Pluripotent	0.69079	0.73238
Mesoderm	0.86610	0.84141
Anterior Endoderm	0.71386	0.79712
Early Endoderm	0.34819	0.51025
Mesendoderm	1.01312	0.22323
Pancreas	0.77519	0.99960
Liver	0.16361	0.38242
Intestine	0.53195	0.55953
Ectoderm	1.17634	0.97005
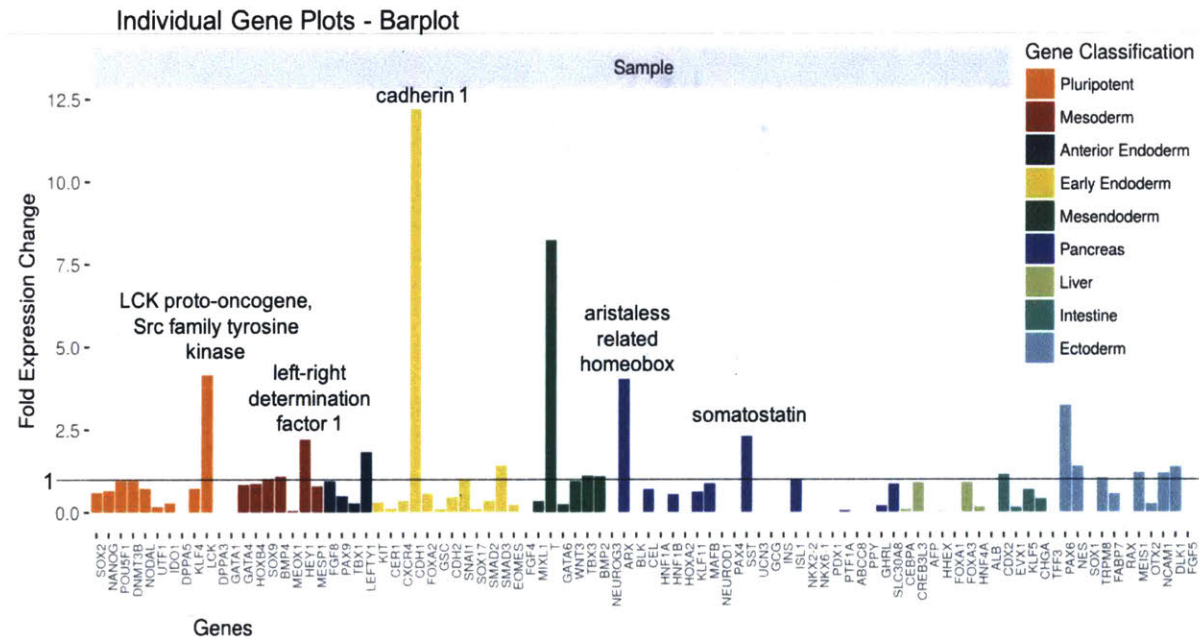
Figure 27. Barplot showing the individual gene expression levels of genes present in each group. Plots are colored by the functional class of the genes and are shown on a log-transformed scale. Significantly upregulated genes in *EXT1*-/- cells are annotated with their full names.

In chemically-undefined strategy, differentiation of hPSCs into a definitive endodermal lineage was induced with PRMI 1640 medium supplemented with Activin A, Wnt3a and B27 minus insulin. Both WT and *EXT1*-/- cells differentiated into SOX17-positive endodermal cells (Figure

28A). Interestingly, *EXT1*^{-/-} cells exhibited a slightly higher endodermal differentiation efficiency than WT with 36.5% SOX17-positive cells in the *EXT1*^{-/-} and only 31% SOX17-positive cells in the WT (Figure 28B, 28C, 28D, 28E). This finding is not totally unexpected given that B27 supplement contains an undefined component-BSA (Bovine serum albumin). Albumin is a highly soluble, acidic protein. It is known that albumin has both high affinity, and secondary binding sites for many anionic, cationic and neutral molecular species [142]. It is safe to assume that BSA in the B27 supplement may have sequestered and introduced HS back to *EXT1*^{-/-} cells during treatment and thereby compensating for any differentiation defect.
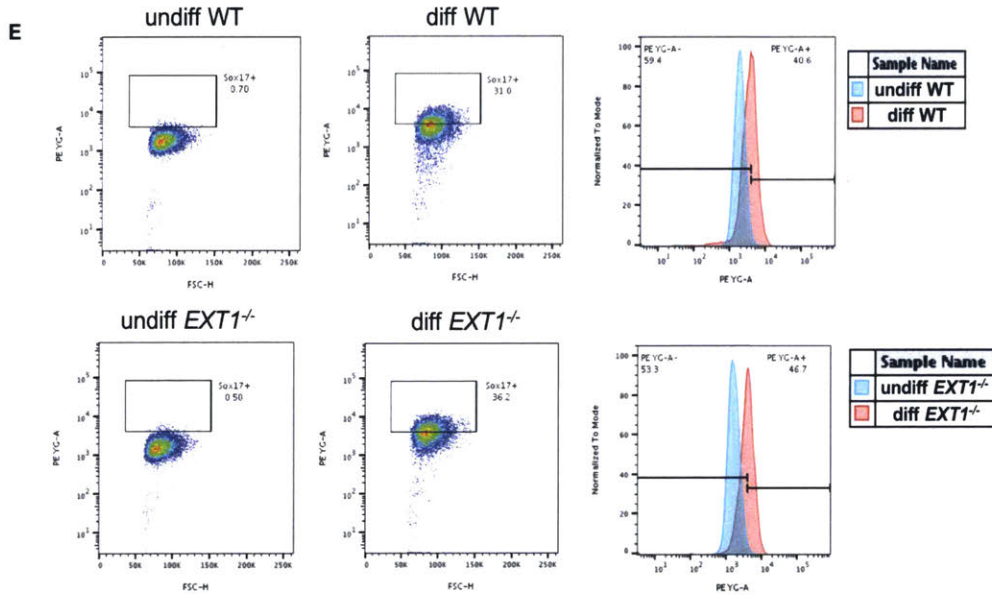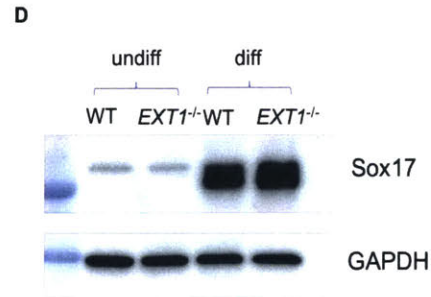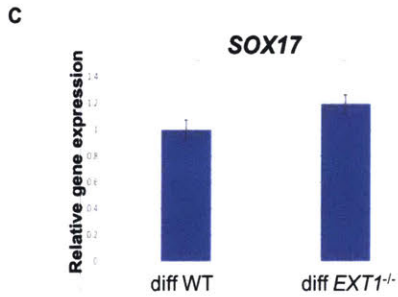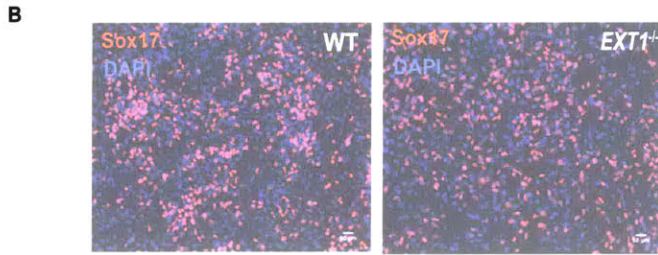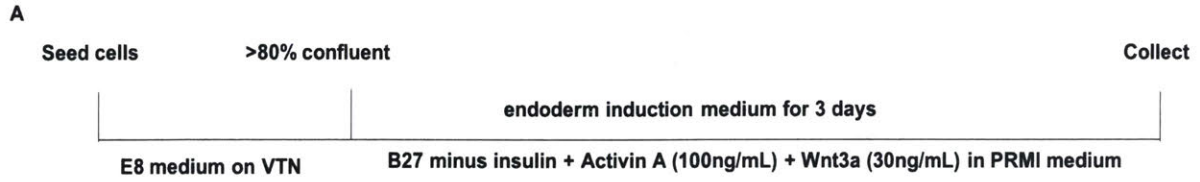
Figure 28. Chemically-undefined endoderm differentiation of WT and *EXT1*^{-/-} cells

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(A) Chemically-undefined endoderm differentiation protocol. Cells were grown to 80% confluency when a cocktail of B27 minus insulin, Activin A and Wnt3a in RPMI was introduced to induce endoderm differentiation. (B) Immunostainings of differentiated WT and *EXT1*$^{-/-}$ cells stained for Sox17 (red) and counterstained for DAPI (blue). (C) qPCR analysis of *SOX17* in WT and *EXT1*$^{-/-}$ cells. (D) Protein level of Sox17 using a polyclonal anti-Sox17 antibody and GAPDH as a loading control. (E) Flow cytometry analysis of Sox17 in WT and *EXT1*$^{-/-}$ cells. The black box represents a gate for Sox17-positive events. A total of 100,000 individual events were collected.

6.2. Summary and perspective

In summary, *EXT1*$^{-/-}$ cells have attenuated ability to differentiate into definitive endoderm under chemically-defined conditions. This observation supports previous findings that *EXT1*$^{-/-}$ cells have impaired ability to differentiate into Brachyury-positive mesendoderm cells. Moving forward, it would be interesting to look into the mechanisms underlying DE differentiation defect. In addition, *EXT1*$^{-/-}$ SOX17-reporter cell line would be useful for the sorting of *EXT1*$^{-/-}$ SOX17-positive and *EXT1*$^{-/-}$ SOX17-negative cells, then one can look into the differentiation potentiality of *EXT1*$^{-/-}$ SOX17-positive to hepatocytes and β cells or do single cell RNA-seq to compare transcriptional changes.

Chapter 7:

Future direction: Understanding the pathology of hereditary multiple exostoses

7.1. Goals and Proposed Experiments

HS acts as an important mediator for the transduction of extrinsic cues into intrinsic signaling pathways [19]. While HS deficiency is linked to many developmental defects in mouse studies, the molecular mechanisms of how HS regulates tissue development or stem cell fate decisions of human embryonic stem cells remain underappreciated. In my opinion, the ultimate goal of this project is to relate stem cell research to human diseases. Bone development defect and nervous system dysfunction are the two most prominent symptoms in patients with HME [57-60]. Moving forward, one should look into how HS affects bone development specially on the proliferation and differentiation of osteoblast and chondrocyte as over-proliferation of chondrocytes and de-differentiation of osteoblasts have been proposed as two potential underlying pathologic mechanisms behind HME [54]. Both osteoblasts and chondrocytes are derived from mesenchymal stem cells (MSCs) [143]. A good experiment to start with would be to investigate whether $EXT1^{-/-}$ cells could differentiate into MSCs, followed up with the induction of osteoblast and chondrocyte differentiation from MSCs. To better mimic the physiological microenvironment, one can even go beyond 2D cell culture to culture and differentiate $EXT1^{-/-}$ cells into bone organoid with three-dimensional (3D) cell culture techniques [144, 145]. Besides $EXT1^{-/-}$ cells, we should also resort to other types of cell lines. For example, we can retrieve primary cells from bone marrow of patients with HME and differentiate them into bone, adipogenic, and chondrogenic lineage in vitro [146]. Since HME is linked to heterozygous loss-of-function mutations in $EXT1$ or $EXT2$ [39], to better mimic the disease processes, it would be useful to generate a $EXT1^{+/-}$ cell line and repeat a similar set of experiments described in this thesis to see whether similar results can be recapitulated in $EXT1^{+/-}$ cells.

In conclusion, the work conducted so far on this project demonstrated that HS plays an active and necessary role in mesendoderm and definitive endoderm differentiation of hESCs by facilitating FGF, Activin A and BMP signaling pathways. Hopefully, these findings will eventually be transformative in the field of biotechnology engineering and clinical applications.

Acknowledgment

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