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Inverse agonism of retinoic acid receptors directs epiblast cells into the paraxial mesoderm lineage

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ABSTRACT

We have investigated the differentiation of paraxial mesoderm from mouse embryonic stem cells utilizing a *Tbx6-EYFP/Brachyury* (*T*)*-Cherry* dual reporter system. Differentiation from the mouse ESC state directly into mesoderm via Wnt pathway activation was low, but augmented by treatment with AGN193109, a pan-retinoic acid receptor inverse agonist. After five days of differentiation, T^+ cells increased from 12.2% to 18.8%, $Tbx6^+$ cells increased from 5.8% to 12.7%, and $T^+/Tbx6^+$ cells increased from 2.4% to 14.1%. The synergism of AGN193109 with Wnt3a/CHIR99021 was further substantiated by the increased expression of paraxial mesoderm gene markers *Tbx6*, *Msgn1*, *Meox1*, and *Hoxb1*. Separate to inverse agonist treatment, when mouse ESCs were indirectly differentiated into mesoderm via a transient epiblast step the efficiency of paraxial mesoderm formation markedly increased. $Tbx6^+$ cells represented 65–75% of the total cell population after just 3 days of differentiation and the expression of paraxial mesoderm marker genes *Tbx6* and *Msgn* increased over 100-fold and 300-fold, respectively. Further evaluation of AGN193109 treatment on the indirect differentiation protocol suggested that RARs have two distinct roles. First, AGN193109 treatment at the epiblast step and mesoderm step promoted paraxial mesoderm formation over other mesoderm and endoderm lineage types. Second, continued treatment during mesoderm formation revealed its ability to repress the maturation of presomitic mesoderm into somitic paraxial mesoderm. Thus, the continuous treatment of AGN193109 during epiblast and mesoderm differentiation steps yielded a culture where ~90% of the cells were $Tbx6^+$. The surprisingly early effect of inverse agonist treatment at the epiblast step of differentiation led us to further examine the effect of AGN193109 treatment during an extended epiblast differentiation protocol. Interestingly, while inverse agonist treatment had no impact on the conversion of ESCs into epiblast cells based on the expression of *Rex1*, *Fgf5*, and pluripotency marker genes *Oct4*, *Nanog*, and *Sox2*, after three days of differentiation in the presence of AGN193109 caudal epiblast and early paraxial mesoderm marker genes, *T*, *Cyp26a1*, *Fgf8*, *Tbx6* and *Msgn* were all highly up-regulated. Collectively, our studies reveal an earlier than appreciated role for RARs in epiblast cells and the modulation of their function via inverse agonist treatment can promote their differentiation into the paraxial mesoderm lineage.

1. Introduction

The differentiation of pluripotent stem cells can be used as a valuable approach to study development, disease and obtain therapeutic progenitor cells. Our interest in skeletal biology has motivated us to learn how to differentiate embryonic stem cells (ESCs) into paraxial mesoderm. The paraxial mesoderm is a highly desirable cell population to generate because it is the precursor that gives rise to all of the cartilage, bone, skeletal muscle, and tendons that comprise the axial skeleton.

An increasing body of work has provided crucial insight into the mechanisms that instruct paraxial mesoderm formation. Two transcription factors, *T-box 6* (*Tbx6*) and *Mesogenin* (*Msgn*) are pivotal regulators in the transition of caudal epiblast cells into unsegmented paraxial mesoderm, which is also known as presomitic mesoderm. Targeted loss of *Tbx6* in mice resulted in the formation of ectopic neural tubes at the expense of paraxial mesoderm formation (Chapman and Papaioannou, 1998). Tbx6 represses *Sox2* expression to direct caudal epiblast cells into the paraxial mesoderm lineage (Nowotschin et al., 2012; Takemoto et al., 2011). Msgn orchestrates the differentiation and migration of progenitor cells exiting the tailbud which form the presomitic mesoderm, and is therefore critical for balancing paraxial

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mesoderm specification and maintenance of the caudal progenitor population (Chalamalasetty et al., 2014; Fior et al., 2012). Together, Tbx6 and Msgn control the differentiation and maturation of paraxial mesoderm required for proper embryo elongation.

Wnt and FGF signaling pathways have essential roles in the lineage specification and outgrowth of axial progenitor cells. Targeted loss of *Wnt3a* (Yoshikawa et al., 1997) and *Lef1/Tcf1* double mutants (Galceran et al., 1999) resulted in a phenotype similar to loss of *Tbx6* in that broader contribution of epiblast cells into the neural ectoderm fate at the cost of forming paraxial mesoderm. With that in mind, there is an overwhelming amount of genetic and biochemical data showing that canonical Wnt signaling plays a major role in mesoderm formation. Gene targeting studies in mice have shown how several members of the Wnt pathway are required for mesoderm formation including, *Wnt3*, *β-Catenin*, and *LRP5/LRP6* double mutants (Huelsken et al., 2000; Kelly, 2004; Liu et al., 1999). During ESC differentiation, Wnt3a and GSK3β antagonists are commonly used to induce mesoderm formation with higher levels of canonical Wnt activity promoting the formation of paraxial mesoderm (Craft et al., 2013; Gadue et al., 2006; Lindsley, 2006; Tanaka et al., 2009; Umeda et al., 2012). FGF signaling is indispensable during embryogenesis, as knockouts of *Fgf4, Fgf8, Fgfr1, or Fgfr2* are all early embryonic lethal (Arman et al., 1998; Deng et al., 1994; Feldman et al., 1995; Sun et al., 1999), and dominant negative mutants display disruption of gastrulation cell movements, lack of mesoderm specification, and severe caudal truncation (Amaya et al., 1991; Fletcher and Harland, 2008). FGF signaling in epiblast cells promotes migration through the primitive streak to initiate mesoderm formation and patterning, and a high FGF concentration persists in the tailbud to maintain the pluripotency and proliferation of cells contributing to the posterior paraxial mesoderm (Boulet and Capecchi, 2012; Burdsal et al., 1998; Ciruna and Rossant, 2001). Subsequent to presomitic mesoderm formation, a cyclical process of maturation occurs that involves periodic segmentation to form paired somites. Two important transcription factors *Mesp2* and *Ripply2* are critical in forming the segmental border at the anterior-most region of the presomitic mesoderm thereby controlling the size and organization of each somite (Takahashi et al., 2010; Zhao et al., 2015). Functioning as negative regulators, *Mesp2* and *Ripply2* establish the anterior boundary of Tbx6, and therefore mark the extent of the presomitic mesoderm domain. A negative feedback loop exists in the PSM wherein Tbx6, along with Notch signaling, induces *Mesp2* expression which in turn induces *Ripply2* expression (Sasaki et al., 2011; Yasuhiko et al., 2008). Mesp2 and Ripply2 cause Tbx6 degradation, while Ripply2 also downregulates *Mesp2* expression to complete somite patterning (Moreno et al., 2008; Wanglar et al., 2014).

The maturation of paraxial mesoderm is also highly regulated by Retinoic Acid (RA) signaling. At the caudal end of the developing embryo, *Cyp26a1*, a gene that encodes for an enzyme which breaks down retinoic acid, is expressed thereby specifying a region absent of RA signaling (Fujii, 1997). Targeted loss of *Cyp26a1* results in severe caudal truncation, which mimics defects caused by teratogenic levels of RA (Abu-Abed et al., 2001; Sakai et al., 2001). Examination of *Wnt3a* and *Fgf8* expression in *Cyp26a1* mutants, genes normally expressed at the caudal end and important for driving axial outgrowth were downregulated. However, as the anterior most region of presomitic mesoderm distances itself from the caudal end where *Cyp26a1* is expressed, levels of RA signaling increase in conjunction with the formation of somites and *Meox1* expression (Haselbeck et al., 1999; Mankoo et al., 2003). *Aldh1a2,* a gene that encodes for an enzyme responsible for RA synthesis is highly expressed in the somites and is necessary for proper somite development (Rhinn and Dolle, 2012). Interestingly, of the three Retinoic Acid Receptors (RARα,β,γ), RARγ and RARβ are expressed in distinct zones that correspond with the caudal tail and trunk region, zones retaining low and high retinoic acid signaling, respectively.

In this study, we have undertaken efforts to direct mouse ESCs into paraxial mesoderm. Our work has led us to become very interested in the role of RARs in this process. Here we present evidence that inverse agonism of RARs via treatment of AGN193109, a pan-RAR inverse agonist in epiblast cells and nascent mesoderm promotes their differentiation into paraxial mesoderm.

2. Materials and methods

2.1. Cell culture

Mouse ESCs were maintained on 0.1% gelatin coated tissue culture dishes (Thermo Scientific) and grown in serum-free maintenance media containing a 1:1 mixture of DMEM/F12 and Neurobasal medium (Life Technologies), N2 and B27 supplements, 0.05% BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.5×10^{-4} M monothioglycerol, 3μM CHIR99021 (Stemgent), 1μM PD0325901 (Cayman Chemical), and 10 ng/ml LIF (Millipore) (Nagy et al., 2003). For differentiation, mouse ESCs were seeded on tissue culture dishes coated with Geltrex (Gibco) and grown for a minimum of 24 h in maintenance media. For differentiation, cells were grown in a 3:1 mixture of IMDM and Ham's F12 (Life Technologies) N2, B27 without vitamin A, 0.05% BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.5×10^{-4} M monothioglycerol, and 0.5 mM ascorbic acid. For mesoderm differentiation, 50 ng/ml Wnt3a (PeproTech), 3uM CHIR99021 (Stemgent), 1μM AGN193109 (Santa Cruz Biotechnology), 10 ng/ml FGF2 and 100 ng/ml Noggin (PeproTech) were added in different combinations. Epiblast induction was carried out in differentiation media containing 10 ng/ml FGF2 and 10 ng/ml Activin A (R&D Systems), with or without 1μM AGN193109 for the indicated durations. *Note:* While initial experiments used both Wnt3a and CHIR99021, it was determined that the potency of this combination was negligible to that of CHIR99021 alone. Therefore, subsequent experiments utilized CHIR99021 without the addition of Wnt3a.

2.2. Generation of fluorescent reporter ESC lines

For the creation of *Tbx6*-EYFP/*T*-Cherry dual reporter mouse ESCs, a BAC clone CTD-2379F21 (Children's Hospital Oakland Research Institute) containing the *Brachyury* gene was engineered with a Cherry fluorescent reporter gene using bacterial recombination strategies as previously described(Gong et al., 2002). In brief, a homology arm was PCR amplified from the BAC clone using *Pfx* DNA polymerase (Life Technologies) with primers 5′-CTCTGCGGCCGCACTGAATTTCGGTCC CCAGAGA-3′ (sense), 5′-CTCTGGATCCGAAGCCCAGACTCGCTACC TGA-3′ (antisense). The DNA fragment was cloned into the Not1-BamH1 site of pLD53.SC2-Cherry and Rec A was used to integrate the reporter into the BAC clone. The BAC clone was then retrofitted with puromycin resistance through Cre/LoxP recombination by co-electroporating pCTP, which expresses Cre recombinase and pUni, which contains an EF1α-puromycin resistance gene and a LoxP site into CTD-2379F21 competent bacteria. Purified BAC was transfected into Tbx6-H2B-EYFP ESCs (generously provided by Sonja Nowotschin and Katerina Hadjantonakis (van den Brink et al., 2014)) using Lipofectamine 2000 (Life Technologies) and clones were enriched by puromycin selection and screened for the transgene by PCR genotyping using 5′-CTCTGCGGCCGCACTGAATTTCGGTCCCCAGAGA-3′ (sense) and 5′-GCACCTTGAAGCGCATGAACTCCTTGATGA-3 (antisense). Reporter expression was observed in individual clones by in vitro differentiation to select for optimal expressing cell lines.

2.3. FACS sorting and analyses

ESCs were washed twice with cold PBS then digested using Accutase (StemCell Technologies) and centrifuged at 300*g* for 5 min. Cells were then resuspended in FACS staining buffer (PBS, 0.5% BSA, 2 mM EDTA, pH 7.2) and sorted for *Tbx6-EYFP* and *T-Cherry* reporter expression. FACS sorting was carried out using a FacsAria II.